PB80-202427

Investigation of the Persistence and Replication of Nuclear Polyhedrosis Viruses in Vertebrate and Insect Cell Cultures by the Use of Hybridization Techniques

Scripps Clinic and Research Foundation
La Jolla, CA

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

May 80

U.S. Department of Commerce
National Technical Information Service
NTIS

PB80-202427

United States
Environmental Protection
Agency

Industrial Environmental Research
Laboratory
Research Triangle Park NC 27711

EPA-600/1-80-026
May 1980

Research and Development

EPA

Investigation of the Persistence and Replication of Nuclear Polyhedrosis Viruses in Vertebrate and Insect Cell Cultures by the Use of Hybridization Techniques

RESEARCH REPORTING SERIES

Research reports of the Office of Research and Development, U.S. Environmental Protection Agency, have been grouped into nine series. These nine broad categories were established to facilitate further development and application of environmental technology. Elimination of traditional grouping was consciously planned to foster technology transfer and a maximum interface in related fields. The nine series are:

1. Environmental Health Effects Research
2. Environmental Protection Technology
3. Ecological Research
4. Environmental Monitoring
5. Socioeconomic Environmental Studies
6. Scientific and Technical Assessment Reports (STAR)
7. Interagency Energy-Environment Research and Development
8. "Special" Reports
9. Miscellaneous Reports

This report has been assigned to the ENVIRONMENTAL HEALTH EFFECTS RESEARCH series. This series describes projects and studies relating to the tolerances of man for unhealthful substances or conditions. This work is generally assessed from a medical viewpoint, including physiological or psychological studies. In addition to toxicology and other medical specialities, study areas include biomedical instrumentation and health research techniques utilizing animals — but always with intended application to human health measures.

This document is available to the public through the National Technical Information Service, Springfield, Virginia 22161.

EPA-600/1-80-026
May 1980

INVESTIGATION OF THE PERSISTENCE AND REPLICATION OF NUCLEAR POLYHEDROSIS VIRUSES IN VERTEBRATE AND INSECT CELL CULTURES BY THE USE OF HYBRIDIZATION TECHNIQUES

by

William Meinke, D. A. Goldstein, Cynthia Alvidrez, and John Spizizen

Scripps Clinic and Research Foundation
10666 N. Torrey Pines Road
La Jolla, California 92037

and

C. B. William and H. R. Lukens

IRT Corporation
7650 Convoy Court
San Diego, California 92138

Contract No. 68-02-2209

Project Officer

C. Y. Kawanishi

Environmental Toxicology Division
Health Effects Research Laboratory
U.S. Environmental Protection Agency
Research Triangle Park, North Carolina 27711

HEALTH EFFECTS RESEARCH LABORATORY
OFFICE OF RESEARCH AND DEVELOPMENT
U.S. ENVIRONMENTAL PROTECTION AGENCY
RESEARCH TRIANGLE PARK, NORTH CAROLINA 27711

DISCLAIMER

This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and Approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis, and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The majority of the registered pesticides are chemical agents. A few, however, are biological in nature because the active ingredients are microbial. Of these micro-organisms, viruses are perhaps the most unique in structure, biology, and the intimacy of their parasitic relationship with their hosts. This report considers whether potential biohazards to human health and other biological components of the environment exist when insect viruses are used as pesticides.

F. Gordon Hueter, Ph.D.
Director
Health Effects Research Laboratory

ABSTRACT

The goal of this research is to determine whether a nuclear polyhedrosis virus (NPV) can interact with a variety of mammalian and insect cells in vivo, producing either apparent or inapparent infection. The use of DNA-DNA hybridization techniques to detect the presence of viral genomes in cells will provide a sensitive method of monitoring viral persistence or replication, even in the absence of cytopathic effects or the production of infectious virus particles. It is proposed that the DNA-DNA hybridization technique be developed as a sensitive assay for the replication or persistence of NPV in vitro, allowing a more effective evaluation of the safety of these viruses as biological control agents than has been possible employing previously established methods.

CONTENTS

Foreword iii
Abstract iv
Figures vi

1. Introduction 1
2. Background 2
3. Infectious Unit 4
   Characterization of the tissue culutre infectious unit from Trichoplusia ni (T. ni) cells infected with A. californica NPV . . . 4
4. Materials and Methods 5
   Cells 5
   Virus 5
   Tritium labeling of viral DNA in vivo 5
   Purification of ^{3}H-labeled virions 6
   Infection of monkey kidney cells (CV-1) 7
   Detection of PIB protein by indirect immunofluorescent antibody staining 7
   Electron microscopy 7
5. Results 9
   Defining the infectious unit 9
   Infection of monkey cells with A. californica NPV 19
6. To Determine Whether Free, Non-Occluded Autographica Californica Nuclear Polyhedrosis Virus can Adsorb and Penetrate Mammalian Cells in vitro 26
   Preparation of ^{3}H-labeled free non-occluded A. californica virions in T. ni cells in vitro (used for infection of monkey cells) 26
   Infection of monkey kidney cell cultures with ^{3}H-labeled A. cal. non-occluded virions 28
   Persistence of A. californica DNA in infected CV-1 monkey cells . 31
   Preparation of in vitro labeled A. cal. NPV DNA 33
   In vitro labeling of A. cal. NPV DNA employing E. coli DNA polymerase 35
7. The Use of DNA-DNA Hybridization for Detection of the Persistence of Viral Genomes 38
   Isolation of viral DNA 38
   Preparation of in vitro labeled viral DNA 38
   Inspection of cell lines 42
   Isolation of cellular DNA 42
   DNA reassociation 42
8. Summary 45
9. References 46

FIGURES

Number		Page
1	Purification of ^{3}H-labeled A. californica virus infected T. ni cells 48 hours post-infection	6
2	Incorporation of ^{3}H-thymidine into non-occluded A. californica NPV	10
3	Velocity sedimentation of virions in sucrose gradients	12
4	Velocity sedimentation analysis of virions treated with DNase and detergents	13
5	Particles from sucrose gradients (rod-shaped "bags") with an average length of about 317 nm and a diameter between about 40 and 80 nm with PTA staining	15
6	Occluded dark-staining virus rods in sections of polyhedra which are about 240 to 270 nm	16
7	Virus observed by uranyl acetate staining show "bags" which appear to be "exploding" at both ends	17
8	Virus observed by uranyl acetate staining show "bags" which appear to be "exploding" at both ends	18
9	Alkaline degradation of ^{3}H-thymidine labeled A. californica polyhedral inclusion bodies	19
10	Morphological characteristics typical of polyhedra	21
11	Morphological characteristics typical of polyhedra	22
12	Bizarre morphology of monkey cells maintained at 28°C	23
13	Fluorescence in nuclei of infected T. ni cells	24
14	Control for T. ni cell fluorescence experiment	24
15	Velocity sedimentation of A. cal. virions in a sucrose gradient	27
16	Purification of ^{3}H-labeled A. cal. non-occluded NPV	28

FIGURES (Continued)

Number		Page
17	Uptake of radioactive A. cal. virions by CV-1 monkey cells and T. ni insect cells	29
18	Sedimentation of an SDS lysate of CV-1 monkey cells six hours following infection by ^{3}H-labeled A. cal. virions	30
19	Sedimentation of A. cal. NPV DNA extracted by alkali and SDS from purified PIB	35
20	CsCl-dye equilibrium centrifugation of A. cal. viral DNA	39
21	In vitro labeling of viral DNA	40
22	Alkaline sucrose gradient centrifugation of in vitro labeled A. cal. DNA	41
23	Schematic of extraction of cellular DNA	43
24	Hybridization of in vitro labeled A. cal. DNA	44

SECTION I

INTRODUCTION

This is a combined final report prepared under Contract 68-02-2209 by IRT Corporation for the Environmental Protection Agency. The first part of the work was previously published as IRT report INTEL-RT 8135-001, March 1977 and the second part was published as IRT 8135-010 in January 1978.

This work was carried out in the Department of Microbiology, Scripps Clinic and Research Foundation, and the authors are indebted to Dr. John Spizizen, Chairman, for his support and encouragement.

Much of the experimental work reported was carried out by Robert Mandel with considerable care and skill, and his contribution is hereby acknowledged.

The authors are indebted to Dr. Vail of the USDA, Phoenix, Arizona, and Dr. Timothy Kurtti for provision of materials.

SECTION 2

BACKGROUND

The rapid rise in world population and the subsequent demand for increased food production has given great impetus to the development of safe and effective pesticides. At the same time, the prolonged use of many chemical pesticides since World War II has resulted in the development of insect populations resistant to the effects of many of these pesticides which can harm nontarget organisms and result in the accumulation of toxic chemical residues in the environment.

These problems have focused attention on the possibility of using arthropod viruses as insect pesticides (Ref 2), especially the nuclear polyhedrosis (NPV) and granulosis (GV) viruses. A report from the World Health Organization (WHO) lists more than 400 viruses which are pathogens for insects and mites (Ref 3). These viruses are extremely virulent for their insect hosts, but appear to have a narrow host range, generally limited to a single genus or species (Ref 2). Viral pesticides thus seem to offer the promise of control of defined insect pests without damaging other populations or leaving toxic residues.

Before being licensed for widespread use as a pesticide, an insect virus must be evaluated for any possible harmful effects to insects other than the target species, other invertebrates, vertebrates (including man), and plants. Indirect evidence accumulated during prolonged exposure of workers to NPV during commercial production of the virus, field application, or ingestion in food indicate that NPV induced no clinical symptoms or immunological response in exposed individuals (Ref 4). Numerous other *in vivo* studies have indicated that arthropod NPV cause no deleterious effects on bacteria, plants, invertebrates other than insects, and vertebrates, including numerous fish, avian, and mammalian species (Refs 4-7). Inocula ranged from ten to 1,000 times the normal field exposure of the virus, and the test species were observed for factors including virally induced immunological response, toxicity, pathogenicity, teratogenicity, and carcinogenicity.

The *in vitro* specificity of NPV has also been investigated. The *Autographa californica* (*A. cal.*) NPV has been shown to be able to cross species lines and replicate in cells from several different genera of *Lepidoptera* (Refs 8,9). Attempts to infect vertebrate cell cultures with NPV have been unsuccessful with one exception. Himeno *et al.* (Ref 10) reported the formation of polyhedral inclusion bodies (PIB) after inoculation of FL (human amnion) cells with viral DNA from *Bombyx mori* NPV. Ignoffo and Rafajko (Ref 11) could demonstrate no replication or cytopathic effects (CPE) of the NPV of *Heliothis zea* in four primate cell lines. A later study by McIntosh and Maramorosch (Ref 12) reported no deleterious effects of *H. zea* NPV infection in five human cell lines inoculated with either PIB or virions released from PIB by mild alkali treatment. Three of these cell lines did retain infectious NPV for up to four weeks after inoculation.

These in vitro studies were designed to observe productive NPV infection by detecting the formation of infectious progeny virus or cytopathic effects such as cell rounding and PIB formation. A latent viral infection, or one proceeding without demonstrable CPE, would not be apparent.

Ignoffo et al. (Ref 13) have demonstrated that H. zea NPV can be passed through an established line of H. zea cells without producing free virions or characteristic inclusion bodies. Cell cultures were inoculated with viral DNA and carried through seven consecutive cell passages. After seven cell passages, infectivity, measured by injection of cotton bollworm larvae with culture extracts and subsequent production of PIB and larval death, increased 10^8 times over that present in the original inoculum. Inclusion bodies or virions were not detectable by electron microscopy in any of the passages.

Two strains of Trichoplusia ni (T. ni) NPV have been isolated by plaque morphology (Ref 14). One strain forming 30 or more polyhedra per nucleus was designated MP (many polyhedra) while another producing less than ten polyhedra per nucleus were classified as FP (few polyhedra). Serial passage of MP virus through tissue culture cells led to the production of virus with the FP phenotype. The FP viral inclusions were not lethal to larvae but displayed superior growth potential in vitro. FP virions were released from in vitro cells more quickly and rose to higher titers than the MP viral strain. Serial in vitro passage of this virus seemed to enhance its ability to replicate in vitro, while decreasing the production of PIB and making the detection of CPE more difficult.

These results suggest that techniques capable of determining the fate of the viral genome in infected cell cultures are needed in order to accurately determine the host range of NPV. The finding that viral genes are readily degraded in infected cell cultures would support the belief that nuclear polyhedrosis viruses pose no serious threat to vertebrates, including man and domestic animals. However, if viral genes persist in infected cells, the possibility that certain insect viruses may be potentially hazardous would have to be considered.

The fate of viral gene sequences in infected cell cultures can be followed by the use of extremely sensitive DNA-DNA hybridization techniques (Refs 15,16). The sensitivity of DNA-DNA hybridization is great enough to allow the detection of less than one viral genome equivalent per 5-10 cells. In this procedure purified viral DNA is labeled with radioactive nucleotides in vitro, denatured, and allowed to reassociate in the presence of a vast excess of unlabeled DNA extracted from infected cells. The presence of gene sequences homologous to viral genes in cellular DNA will increase the rate of reassociation of the radioactively labeled viral DNA probe. The number of viral genome equivalents present in the DNA from infected cells can be estimated from the change in the reassociation rate (Ref 17).

SECTION 3

INFECTIOUS UNIT

CHARACTERIZATION OF THE TISSUE CULTURE INFECTIOUS UNIT FROM *Trichoplusia ni* (*T. ni*) CELLS INFECTED WITH *A. californica* NPV

While nuclear polyhedrosis viruses have been propagated successfully *in vitro* in insect tissue culture cells, unequivocal identification of the infectious unit has not been achieved. The original inocula are usually derived from the hemolymph of infected larvae. Once the infection is established, the virus is subsequently propagated by infecting cultures with the overlay media from previously infected cultures. Although it has been generally conceded that polyhedra inclusion bodies (PIB) obtained from tissue culture cells have a low (or no) specific infectivity, it is not known whether the actual infectious unit in the media is enveloped virions, naked virus rods, sub-virion complexes, or naked DNA.

Since our ultimate goal is to detect infection of mammalian cells with insect viruses, it is essential that the infectious entity be defined. To this end, a number of experiments have been performed to isolate and characterize the infectious material from overlay media, as well as from infected cells. The basic approach has been to tag the virus by growing infected cells in the presence of radioactive precursors to DNA, and to fractionate and characterize specific sub-cellular and extra-cellular fractions. Various fractions were characterized by: (1) velocity sedimentation rate; (2) bouyant density of fixed and unfixed material in CsCℓ; (3) sensitivity to various enzymes and detergents; (4) electron microscopy; and (5) infectivity in *T. ni* cells in tissue culture. Growth media was removed from CV-1 cells, and they were covered with overlay media taken from infected *T. ni* cells which contained the infectious entity. In different cultures, virus was permitted to absorb for one hour at one of two temperatures, either 28°C or 37°C. The reason for infecting at 28°C is that this is the optimum growth temperature for virus. The rationale for infecting at 37°C was that this is the optimum growth temperature for the monkey cells. Cells were then overlayed with fresh monkey cell growth media and the cultures monitored for signs of infection. These included examination by immunofluorescence for the presence of virus-specific proteins and observation for cytopathic effect by phase and electron microscopy. Attempts to follow the uptake of radioactive virus were unsuccessful in this initial series of experiments, since it was not possible to achieve a high enough incorporation of radioactivity into virions to make this practical. This approach is now being repeated in experiments in progress.

SECTION 4

MATERIALS AND METHODS

CELLS

Monkey kidney cells (CV-1) were obtained from the American Type Tissue Culture Association, and were grown as monolayer cultures in plastic petri dishes (100 mm). Growth media was Dulbecco's Modified MEM (Gibco), supplemented with 10 percent fetal bovine serum (Gibco) and incubated at 37°C in a 5 percent CO_2 atmosphere. Cells were routinely subcultured by trypsinization, and replated at a 1:3 or 1:4 dilution every four to five days.

Insect cells T. ni (TN-368), were supplied by Dr. W. Fred Hink. Cells were grown in 25 cm^2 Falcon plastic screw cap flasks in Hink's modification of Grace's Insect T.C. medium, the medium designated TNM-FH. The antibiotics, penicillin (Lilly) 50,000 units/100 mℓ and 10 mg/100 mℓ streptomycin SO_4 (Lilly) were added immediately before use. Cells were grown at 28°C and routinely subcultured at a 1:5 dilution every three days by removing the spent growth media, resuspending the cells (shaken off the flask by violent agitation) into fresh growth media, and dispensing the cells into new flasks.

VIRUS

The original A. californica nuclear polyhedrosis virus (NPV) was provided by Dr. W. Fred Hink and consisted of growth media obtained from infected T. ni cells. Subsequent inocula were prepared by harvesting growth media from infected cells and removing high molecular weight material by centrifugation at 5,000 x g. This supernatant fluid containing the infectious unit was stored at 4°C or -70°C.

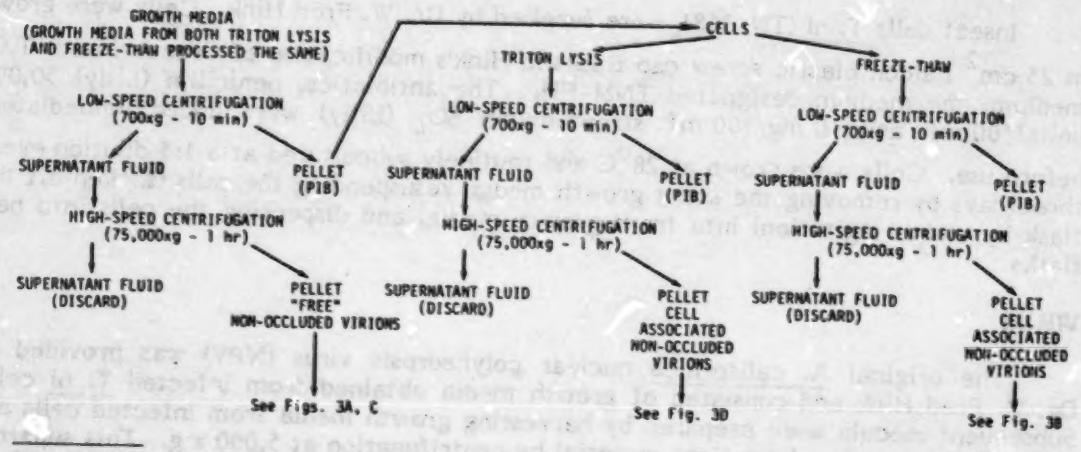
TRITIUM LABELING OF VIRAL DNA in vivo

Viral DNA was labled by the following procedure. T. ni cells were infected in the logarithmic growth phase (70 to 80 percent confluent) by removing the media and inoculating with 0.5 mℓ of media containing infectious virus. This inoculum was a 1:10 dilution of a typical "growth media" inoculum described above. After one hour at 28°C (standard infection procedure), virus was removed and fresh media added containing either (1) 10 μCi/mℓ ^{3}H-deoxycytidine (110 mCi/mmole); (2) 10 μCi/mℓ ^{3}H-thymine (15 Ci/mmole); (3) 10 μCi/mℓ, ^{3}H-deoxycytosine-5'-monophosphate (25.3 Ci/mmole); or (4) 20 μCi/mℓ ^{3}H-thymidine (52.6 Ci/mmole) (New England Nuclear). Cells were maintained at 28°C, and harvested 48 hours after infection.

PURIFICATION OF ^{3}H-LABELED VIRIONS

Non-Occluded "Free" Virions

Growth media containing tritium-labeled precursors as described above (e.g., either thymine, thymidine, deoxycytidine, or deoxycytidine monophosphate) were processed by centrifugation at low speed (700 x g, 10 minutes, 4°C) to remove cell debris, and then centrifuged at high speed (75,000 x g, 60 minutes, 23°C) to pellet the "free" (as opposed to cell-associated) non-occluded virus. The pellet was resuspended in a small volume of sucrose gradient buffer (SGB) (0.05 M Tris, pH 8.0, 0.3 M NaCℓ) layered onto a linear 5.0 mℓ, 10 to 40 percent (wt/wt) sucrose gradient (in SGB) and centrifuged at 24,000 rpm for 30 minutes at 23°C in a Spinco SW 50.1 rotor. Fractions were collected and assayed for radioactivity. Some gradients also contained ^{14}C-labeled T_4 bacteriophage added as a sedimentation marker. This procedure is summarized in Figure 1.



PIB = POLYHEDRAL INCLUSION BODIES.

*Growth media was removed and "free" non-occluded virions purified, as indicated in the flow diagram. In two different experiments, cell-associated virons were extracted by either (1) lysing cells with non-ionic detergent Triton X-100, or (2) by freezing and thawing the cells three times. Further purification was performed as indicated.

Figure 1. Purification of ^{3}H-labeled A. californica virus infected T. ni cells 48 hours post-infection*

Purification of Cell-Associated, Non-Occluded ^{3}H-Labeled Virions

Non-occluded ^{3}H-labeled virions were liberated from cells (after removing ^{3}H-labeled growth media 48 hours after infection) by two different procedures. In one, cells were suspended in a small volume of buffer and subjected to three cycles of rapid freezing and thawing. In the other, cells were suspended in a buffer containing the non-ionic detergent, Triton X-100, designated "Triton lysing fluid" (TLF) (0.25 percent Triton X-100, 0.01 M Tris, 0.01 M EDTA, pH 7.9). After swelling the nuclei for

10 minutes at room temperature in 0.9 mℓ TLF, 0.1 mℓ of 2.0 M NaCℓ was added to each petri plate (final concentration 0.2 M), and incubated another 10 minutes at room temperature. Both the TLF and the "freeze-thaw" lysates were given a low speed centrifugation for 10 minutes at 700 x g at 4°C. The pellets, which contain the polyhedral inclusion bodies (PIB) and cell debris, were processed by treating with detergents (DOC and SDS), and by successive equilibrium centrifugations in 1.0 to 2.2 M sucrose solutions to purify the PIB (see previous reports). The supernatant solutions were processed as described above for purification of "free" non-occluded virions. The purification procedure is summarized in Figure 1.

INFECTION OF MONKEY KIDNEY CELLS (CV-1) WITH A. californica NPV

Monkey cells (CV-1) grown in monolayers (90 percent) confluence on petri plates (100 cm) were infected with A. californica NPV by inoculating each plate with 1.0 mℓ of virus containing insect media (10^8 PFU/mℓ) diluted 1:1 in Dulbecco's MEM media. Infections were carried out at both 28°C and 37°C. After a two-hour adsorption period, cells were overlaid with Dulbecco's MEM supplemented with glutamine and 5 percent horse serum (Gibco). After 24 hours, cells were scraped from some of the plates and processed for electron microscopy. The remaining cultures were routinely subcultured every five to six days. Prior to subculture, media was removed and stored at -20°C for later studies of infectivity.

DETECTION OF PIB PROTEIN BY INDIRECT IMMUNOFLUORESCENT ANTIBODY STAINING

Monkey and insect cells were prepared for fluorescent antibody staining by seeding them in 10 x 35 mm petri plates containing glass coverslips. Cells were infected with 0.2 mℓ of inoculum as usual, and overlaid with 2 mℓ of media. After 48 hours, coverslips were washed three times in phosphate-buffered saline (PBS), and fixed by adding 2 mℓ of ether-ethanol (95 percent), 1:1 for 10 minutes at room temperature. The ether-ethanol was removed and the cells fixed with 95 percent ethanol for 20 minutes. In some cases, the cells were given a 10 minute wash with our standard alkaline PIB dissolution buffer (0.05 M Na_2CO_3, 0.05 M NaCℓ). Cells were again washed in PBS, covered with 0.1 mℓ of rabbit anti-A. californica NPV anti-sera (donated by Dr. Phillip Norton) (1:10 dilution), and incubated for 60 minutes at 37°C. Cells were washed three times in PBS and covered with 0.1 mℓ of goat anti-rabbit fluorescein-conjugated IgG (7S fraction, ICN Biochemicals) (1:10 dilution) and incubated for 60 minutes at 37°C. Cells were washed three times in PBS and mounted face down in glycerol-PBS (9:1) on glass slides. Slides were stored at 4°C until examination with a fluorescence microscope.

ELECTRON MICROSCOPY

Thin Sections

Cells were centrifuged at 600 x g, washed two times in PBS, and fixed in 2 percent phosphate-buffered glutaraldehyde. The cells were suspended in 1 percent osmium tetroxide (one hour) before dehydration and embedding. Sections were stained with 2 percent uranyl acetate and 0.5 percent lead acetate.

Negative Staining of Virions

Virions were fixed in 2 percent phosphate-buffered glutaraldehyde from one to three hours, then dialyzed against distilled water. Drops were placed on paraffin blocks and collodion carbon-coated copper grids placed face down on the drops for three minutes. Grids were removed, and the edge touched to filter paper to remove excess solution. They were then placed on drops of 2 percent uranyl acetate or 2 percent PTA for one to six minutes, blotted, and dried. Grids were examined in a Hitachi HU-11 electron microscope. Magnifications were determined from a grid of a silicon-coated, cross-hatched grating with 54,864 lines/inch.

SECTION 5

RESULTS

DEFINING THE INFECTIOUS UNIT

Incorporation of Tritiated Thymidine into A. californica NPV

To determine the optimum labeling conditions for virus, eight infected petri plate cultures were overlaid with radioactive thymidine (methyl-^{3}H) and incubated at 28°C. At various times, two cultures were harvested, the growth media removed, and "free non-occluded virus" purified (see Figure 1). The cells were lysed by repeated freeze-thaw and the "cell-associated, non-occluded virus" purified (Figure 1). Samples were removed and assayed for the amount of radioactivity by scintillation counting. The results are shown in Figure 2. From this figure it can be seen that radioactivity accumulated most rapidly between about 24 and 40 hours after infection, and thereafter appeared to level off. Between 40 and 48 hours, the amount of virus in cell-associated virus actually decreased, and this may be due to rapid incorporation into polyhedral inclusion bodies. From these data it appears that between 40 to 48 hours is the optimum time period to harvest cells and media for the maximum incorporation of ^{3}H-thymidine into non-occluded virions.

Incorporation of Various DNA Precursors Into NPV

Since it has been reported that thymidine is not efficiently incorporated into NPV when grown in larvae, several other pyrimidine precursors of DNA were tested in tissue culture cells to determine the most suitable precursor for ^{3}H incorporation. These were deoxycytidine, thymine, deoxycytidine-5'-monophosphate, and thymidine. Precursors were added to infected cells immediately after infection, cells and media harvested at 48 hours, and the various fractions assayed for radioactivity. The results are summarized in Table 1. These data show that thymidine is incorporated at the greatest rate, while deoxycytidine is incorporated at the lowest rate. Importantly, the majority of the radioactive virus is incorporated into polyhedra, while less than 10 percent is found in the growth media. Furthermore, the amount of non-occluded, cell-associated virus is three to five times greater than the "free" virions in the growth media.

Velocity Sedimentation Analysis of Labeled Virions

Virions were analyzed by velocity sedimentation through 10 to 40 percent (wt/wt) sucrose solutions. Free non-occluded virions were derived from growth media of T. ni cells 48 hours after infection. Two procedures were used to obtain non-occluded, cell-associated virus. In one, cells were ruptured by freezing, followed by rapid thawing. In

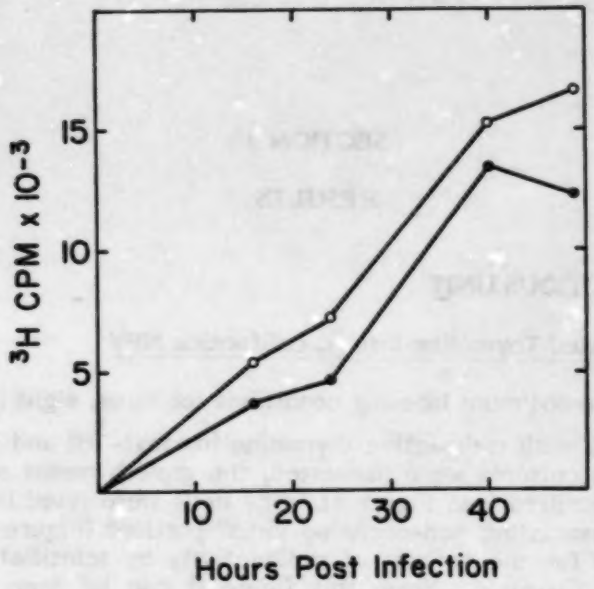


Figure 2. Incorporation of ^{3}H-thymidine into non-occluded A. californica NPV. Following infection of T. ni cells with A. californica NPV (10^8/mℓ), cells were grown in media containing ^{3}H-thymidine (20 μCi/mℓ). At the times indicated, growth media was removed and "free" non-occluded virions purified as indicated by the scheme shown in Figure 1. At the same time, cell-associated non-occluded virions were purified by lysed cells (freeze-thaw procedure, Figure 1). Ordinate scale represents acid-precipitable radioactivity. Open circles, virions in growth media; closed circles, virions from cells.

TABLE I. INCORPORATION OF RADIOACTIVE DNA PRECURSORS INTO A. californica NPV

	Growth Media ("Free" Non-Occluded Petri Dish Virions)		Cells (Cell-Associated/ Non-Occluded Virions)		Polyhedra Inclusion Bodies	
Precursor	CPM/PD mmole x 10^{-8}	% of Total Incorporation	CPM/PD mmole x 10^{-8}	% of Total Incorporation	CPM/PD mmole x 10^{-8}	% of Total Incorporation
Deoxycytidine	5.3	1	18	3	656	96
Thymine	529	7	2,809	36	4,390	57
Deoxycytidine Monophosphate	19,209	4	70,326	15	368,987	80
Thymidine[a]	131,289	-	96,747	-	-[a]	-

[a]Polyhedra were not quantitated in this experiment.

the second, cells were lysed by the non-ionic detergent Triton X-100 (see Section 2). The purification procedures are diagrammed in Figure 1.

Analysis of the virions is shown in Figure 3. The virions in growth media segment with a velocity of about 1000 S relative to ^{14}C-labeled T_4 bacteriophage (Figures 3a and 3c). (NOTE: Correction is made for density differences between T_4 and A. californica NPV.) The peak of virus is found in the lower third of the gradient. However, also evident is material sedimenting at the top of the gradient with a velocity of less than 100 S. As shown in Figure 4, this material is sensitive to deoxyribonuclease. Since this DNA originated from a virus pellet, it is concluded that it represents DNA liberated from virus (see below for more details on stability of virus). Although there are no values reported for the sedimentation rate of A. californica NPV virions, T. ni NPV have a reported sedimentation rate of 860 S, so that the value of 1000 S for A. californica virions is within the expected range for non-occluded virions. Concerning the sedimentation rate for DNA, other NPV DNAs which have been studied have molecular weights of about 100×10^6 daltons and S values of about 60 to 70 S. It is concluded, therefore, that the purification process as performed in this work produces intact virions, but also causes partial disruption of the virions. Experiments are now underway to evaluate improved procedures for purification of free, non-occluded virions.

As shown in Figure 3b, the freeze-thaw procedure for liberating virus from cells disrupts the virions completely, and only DNA is obtained. This would be consistent with reports that virus in growth media loses activity upon freezing and thawing and storage at -20 C.

In contrast, lysis of cells by Triton X-100 gives the same relative proportion of intact virions as is found in the growth media (Figure 3d). This latter procedure is thus relatively effective in obtaining virus from cells; however, again, there is a considerable amount of virus which is broken down to DNA. Importantly, Triton lysis appears to be a valuable method for extracting non-occluded virions from infected cells, since this has not been previously reported. From our experiments, it is now known whether the lysing process iself or subsequent purification is responsible for the certain percentage of virus breakdown.

Physical and Chemical Composition of Virions

Triton lysates and growth media containing infectious virus were treated with chemicals and enzymes and examined in the electron microscope to identify the radioactively labeled virion. The results of the chemical and enzymatic treatments are shown in Figure 4. These results are summarized as follows.

1. The fast sedimenting virion is insensitive to DNase, but the slow sedimenting material is sensitive to the enzyme.
2. Magnesium alone has no effect on either the virion or the DNA; thus, there is evidently no virion-associated nuclease.
3. Deoxycholate (DOC) degraded the virion. A small intermediate peak with an S value of about 300 S, nevertheless, is generated.

4. The ionic detergent sodium dodecyl sulfate (SDS) degrades the virion to free DNA.

5. Virions are insensitive to Triton X-100.

(It is important to note that the 1000 S peak is defined here as the non-occluded virion.)

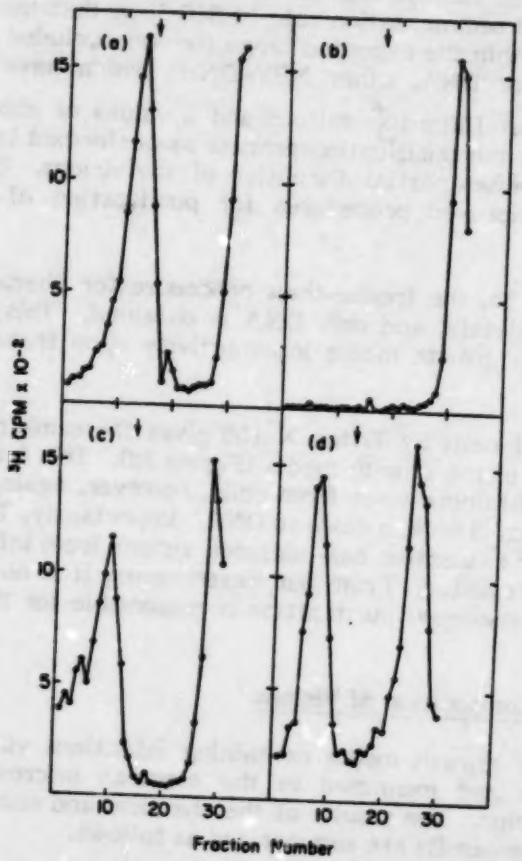


Figure 3. Velocity sedimentation of virions in sucrose gradients. ^{3}H-labeled virions purified as indicated by Figure 1 were concentrated, layered onto 5.0 m 10 to 40% sucrose gradients and centrifuged for 30 minutes at 24,000 rpm in a Spinco SW50.1 rotor at 23°C. Fractions were collected and acid-precipitable radioactivity determined. Growth media (free non-occluded virus) was removed from cells before lysis by either (a) freeze-thaw cycles, or (c) Triton. Cell-associated virus was processed by either (b) freeze-thaw cycles, or (d) Triton lysis. Arrows indicate the position of ^{14}C-labeled T_4 bacteriophage added as a marker. Sedimentation is from right to left.

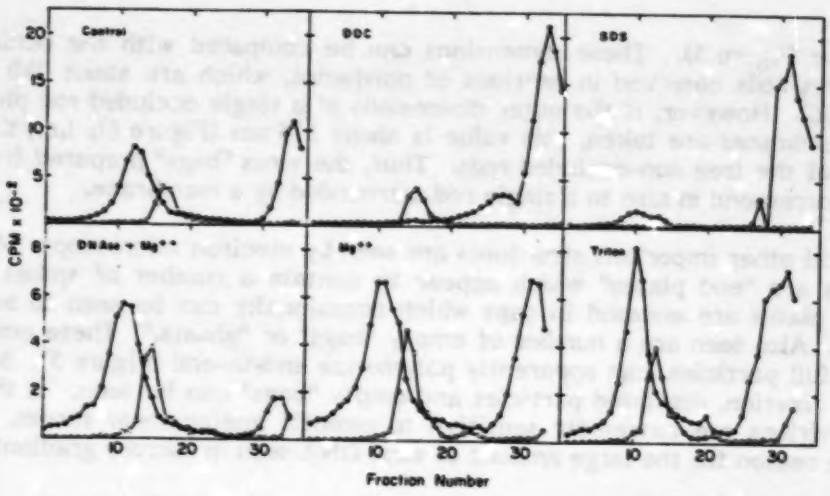


Figure 4. Velocity sedimentation analysis of virions treated with DNase and detergents. Virions were prepared from cells labeled with ^{3}H-thymidine and harvested 48 hours after infection. Virions were incubated for 30 minutes in the presence of the detergents or DNase at 37°C and then sedimented as indicated in the legend to Figure 3. Concentrations used were 0.5% DOC, 1% SDS, 1% Triton, 10 μg/mℓ DNase and 0.01 M Mg^{++}. ^{14}C-labeled T_4 bacteriophage was added as a marker (open circles).

DOC and SDS sensitivity suggests the virion is associated with membranous material, although DOC can also dissociate certain DNA-protein complexes. Triton is known to solubilize cytoplasmic membranes, but nuclear-type membranes are not degraded by this detergent. Perhaps most significant is the fact that the virus, although multiply embedded in polyhedra, is found as a single sedimenting peak in these gradients.

Electron Microscopy of Virions

Peak fractions from sucrose gradients were fixed in glutaraldehyde and virions examined by negative staining with phosphotungstic acid (PTA), or with uranyl acetate (see page 7, Negative Staining of Virions).

The particles from the sucrose gradients appear as rod-shaped "bags," with an average length of about 317 nm and a diameter between about 40 and 80 nm with PTA

staining (see Figure 5). These dimensions can be compared with the occluded dark-staining virus rods observed in sections of polyhedra, which are about 240 to 270 nm (see Figure 6). However, if the outer dimensions of a single occluded rod plus the light staining membranes are taken, this value is about 315 nm (Figure 6); i.e., the same as the length of the free non-occluded rods. Thus, the virus "bags" prepared from sucrose gradients correspond in size to a single rod surrounded by a membrane.

Several other important structures are seen by electron microscopy. At each end of the rods are "end plates" which appear to contain a number of spikes (Figure 5). These end plates are encased in caps which occasionally can be seen to be hinged or ripped out. Also seen are a number of empty "bags" or "ghosts." These empty "bags," as well as full particles, can apparently polymerize end-to-end (Figure 5). Surprisingly, even after fixation, disrupted particles and empty "bags" can be seen. It thus appears that free virions are extremely sensitive to osmotic and/or shear forces, which may explain the reason for the large amount of viral DNA seen in sucrose gradients.

In these electron micrographs, no structure corresponding to the inner dark-staining rod observed in sections of polyhedra is found. Explanation for this may be that the "inner" rods are extremely labile. Actually, virus observed by uranyl acetate staining show "bags" which appear to be "exploding" at both ends (Figures 7 and 8). Within these "exploding" particles are amorphous structures made up of hollow sub-units about 15 nm in diameter. It can be speculated that the inner rods are formed by polymerization of these sub-units.

Infectivity of Virions

Labeled virions were added to both T. ni and monkey kidney cells, and the uptake of radioactive virus monitored. However, the amount of radioactivity was too low to arrive at meaningful conclusions as to the absolute uptake of radioactivity. These experiments are being repeated with virus labeled to very high specific radioactivity. The specific infectivity of these virions is also being determined.

Virions Derived From Polyhedra as Infectious Units

Since "free" and "cell-associated" non-occluded virions only account for about 10 to 30 percent of the total labeled virus in cell cultures (the majority of virions having been occluded into polyhedra), attempts were made to obtain virions from polyhedra by alkaline dissolution. The results of these experiments are shown in Figure 9. The top two panels (Figures 9a and 9b) represent velocity sedimentation in 10 to 40 percent sucrose gradients designed to detect non-occluded virions, while the bottom panels (Figures 9c and 9d) represent equilibrium sedimentation in sucrose (1 to 2.2 M). In the left panels (Figures 9a and 9c), alkali degradation was performed in the presence of the inhibitor of proteases PMSF (phenyl methyl sulfonyl fluoride), since it has recently been reported that polyhedra contain an endogenous protease. However, in all cases, no peaks corresponding to intact virions were found upon alkaline degradation of the polyhedra. At this time, the explanation for failure to obtain virions from polyhedra is not known.

Figure 5. Particles from sucrose gradients (rod-shaped "bags") with an average length of about 317 nm and a diameter between about 40 and 80 nm with PTA staining

Reproduced from
best available copy.

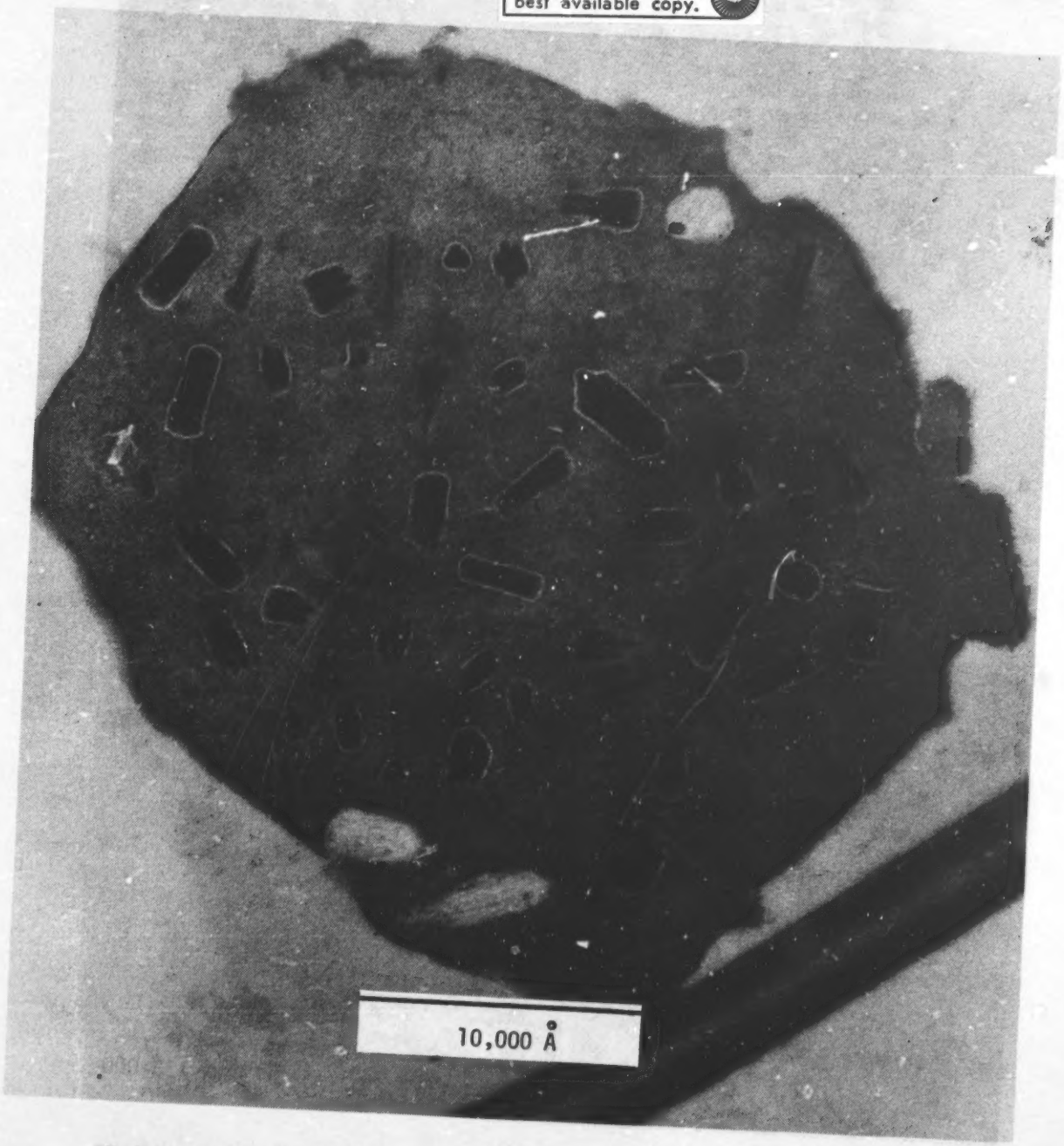


Figure 6. Occluded dark-staining virus rods in sections of polyhedra which are about 240 to 270 nm

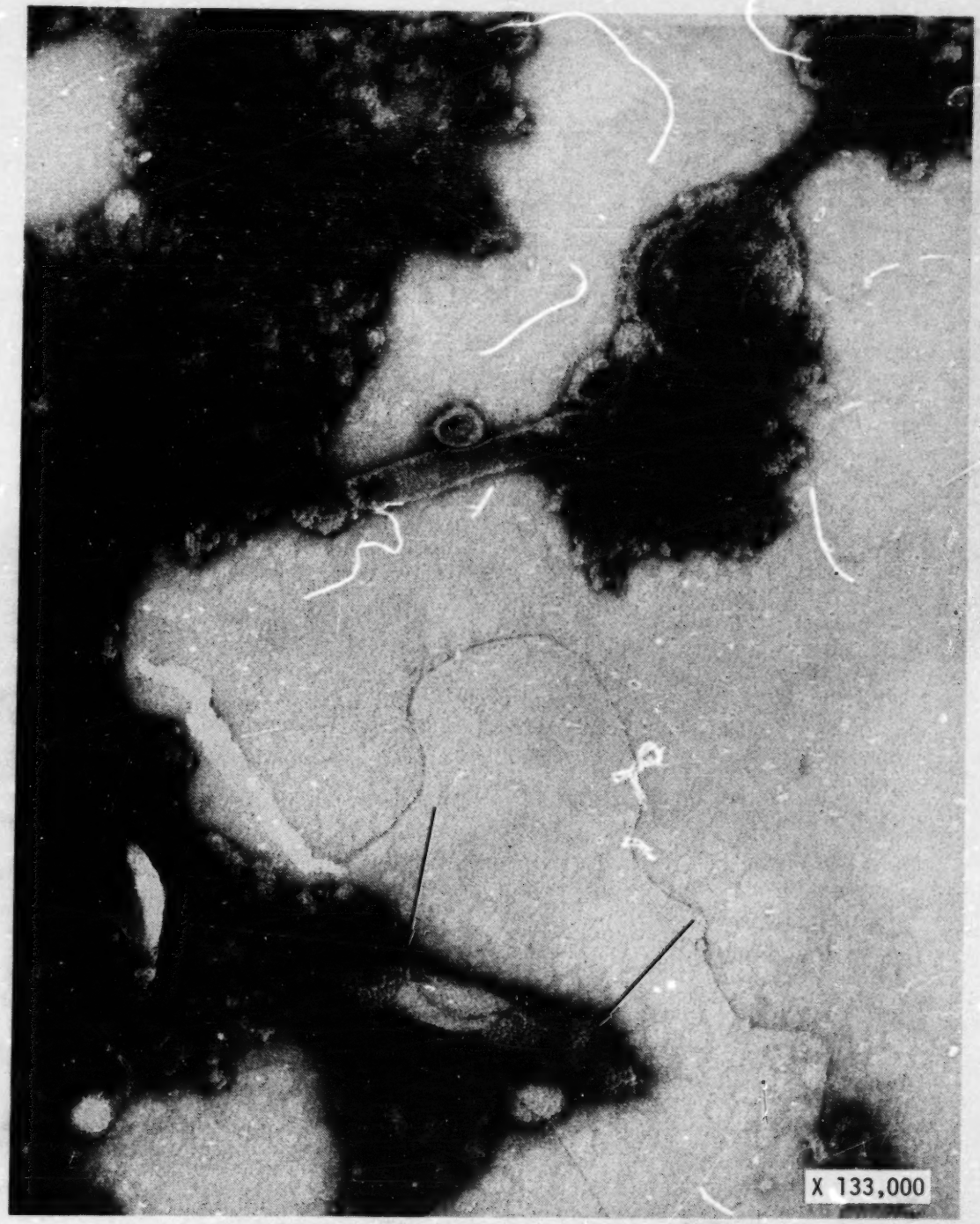


Figure 7. Virus observed by uranyl acetate staining show "bags" which appear to be "exploding" at both ends

Figure 8. Virus observed by uranyl acetate staining show "bags" which appear to be "exploding" at both ends

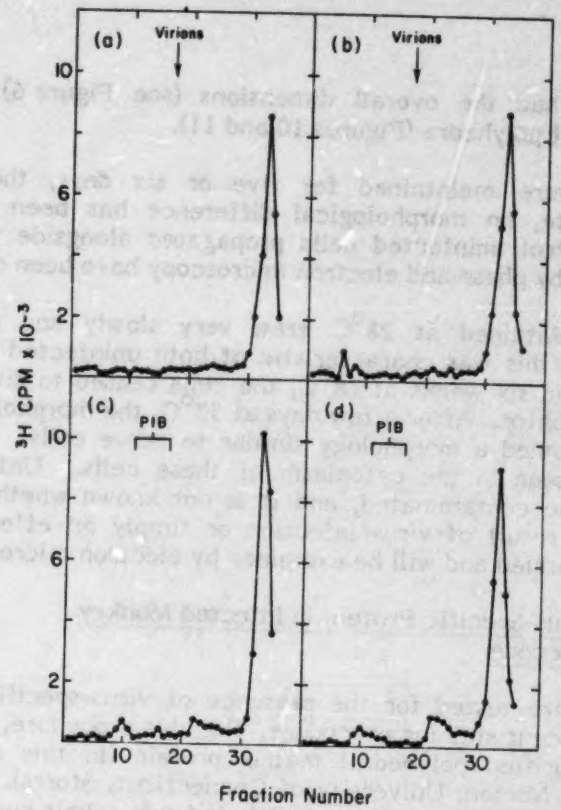


Figure 9. Alkaline degradation of ^{3}H-thymidine labeled A. californica polyhedral inclusion bodies. Polyhedral inclusion bodies were dissolved in pH 10.5 phosphate buffer in the presence (a and c) or absence (b and d) of the protease inhibitor phenyl methyl sulfonile fluoride (0.2%) for two hours at 37°C. Samples were analyzed by velocity sedimentation of 10 to 40% sucrose gradients (a and b) or by equilibrium sedimentation in 1.0 to 2.2 M sucrose (c and d). Arrows and brackets indicate the position of untreated virions and polyhedral bodies, respectively.

INFECTION OF MONKEY CELLS WITH A. californica NPV

Morphology of Infected Cells

Monkey kidney cells (CV-1) were infected with A. californica NPV at 28°C and 37°C (see Section 2). Cells were incubated for 24 and 48 hours, and cells harvested for examination by electron microscopy. Cells were either scraped from the petri plates or trypsinized to remove them.

Electron microscopy failed to reveal any structures which resembled virions or polyhedra, nor was there any evidence that virions had penetrated the cell membrane. Although the CV-1 cells did contain occasional inclusion bodies, none of these were

observed in nuclei or had the overall dimensions (see Figure 6) or morphological characteristics typical of polyhedra (Figures 10 and 11).

Other cultures were maintained for five or six days, then subcultured by trypsinization. To date, no morphological difference has been detected between infected cells and control uninfected cells propagated alongside the infected cells. Repeated examinations by phase and electron microscopy have been carried out.

Monkey cells maintained at 28°C grew very slowly and assumed a bizarre morphology (Figure 12); this was characteristic of both uninfected and infected cells. After three passages and six weeks at 28°C, the cells ceased to divide, and were then placed in the 37°C incubator. After a few days at 37°C, the morphology again changed; this time the cells assumed a morphology similar to nerve cells. A large number of inclusion bodies were seen in the cytoplasm of these cells. Unfortunately, control uninfected cells became contaminated, and it is not known whether the cytoplasmic inclusion bodies are a result of virus infection or simply an effect of temperature. These cells are being carried and will be examined by electron microscopy.

Attempts to Detect Virus-Specific Protein in Infected Monkey Cells by Immunofluorescence

Infected cells were tested for the presence of virus-specific protein using the indirect immunofluorescent staining procedure. For this procedure, antibody is made in rabbits against the purified polyhedral matrix protein (in this study, antibody was provided by Dr. Phillip Norton, University of Connecticut, Storrs). This anti-serum is allowed to react with cells, then fluorescein-labeled anti-rabbit goat immunoglobulin is added to "stain" the antigen-antibody complex. Fluorescent areas within cells are indicative of the presence of viral antigen.

When A. californica NPV anti-serum was reacted with infected CV-1 cells, no immunofluorescence could be detected. In contrast, infected T. ni cells show very bright fluorescence in nuclei corresponding to the polyhedral inclusion bodies (Figure 13), as opposed to the control (Figure 14).

PRELIMINARY SUMMARY

Summarizing the work thus far, it has been shown that an infectious form of the virus, A. californica NPV, could be obtained from the growth media taken from infected insect cells of Tricoplusia ni in culture. This virus was concentrated by centrifugation and purified by velocity sedimentation in sucrose gradients. The virus was identified by its morphology in the electron microscope, sedimentation properties, and infectivity.

Growth media containing free infectious virions was added to CV-1 monkey cells, and the cells observed for signs of cytopathic effect. Observations of cytopathic effect (CPE) were made by phase contrast microscopy as well as by electron microscopy. Infected monkey cells were further tested for the presence of viral matrix protein by indirect immunofluorescence, using antibody prepared in rabbits. Tests for the persistence of virus within the monkey cells were uniformly negative.

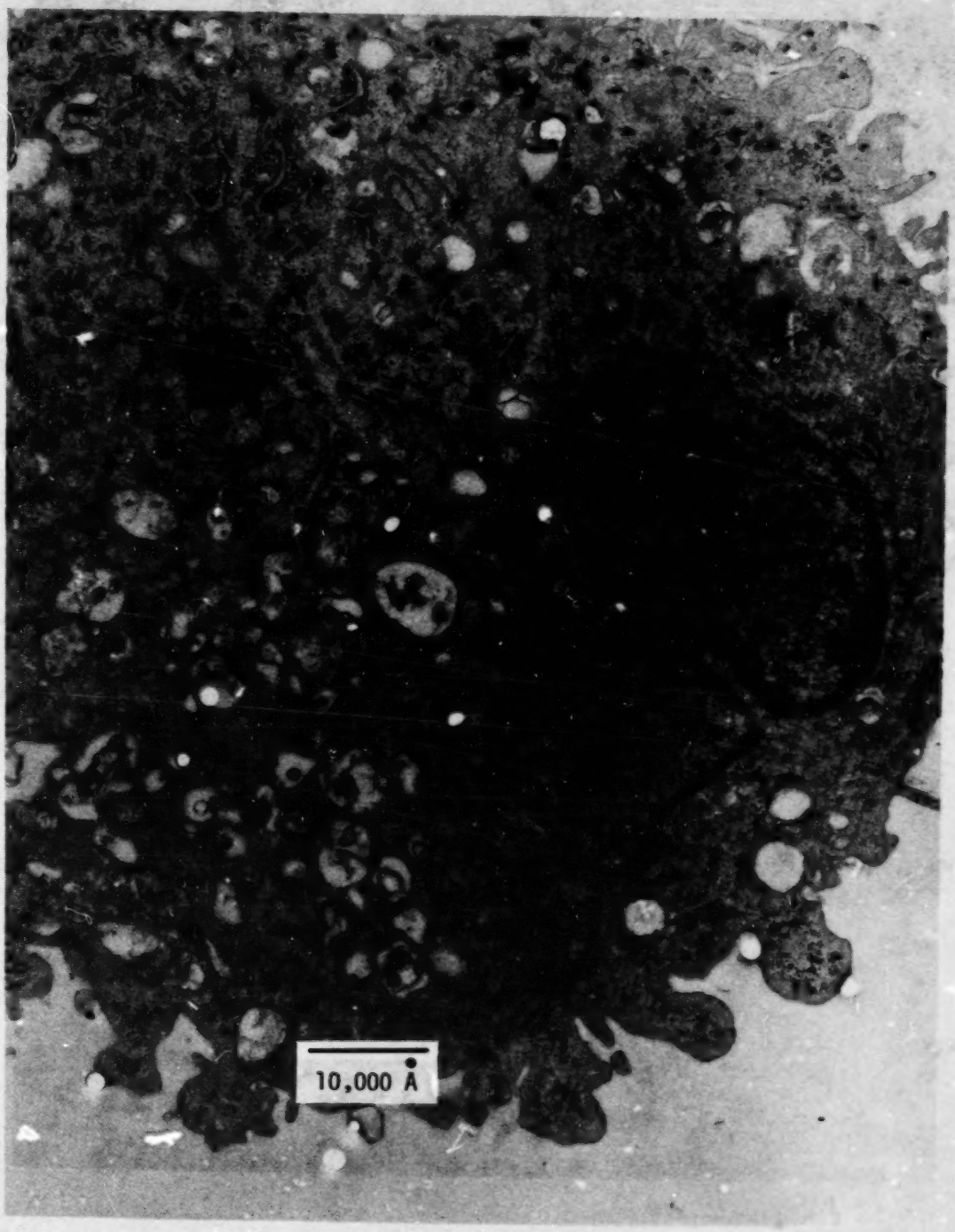


Figure 10. Morphological characteristics typical of polyhedra

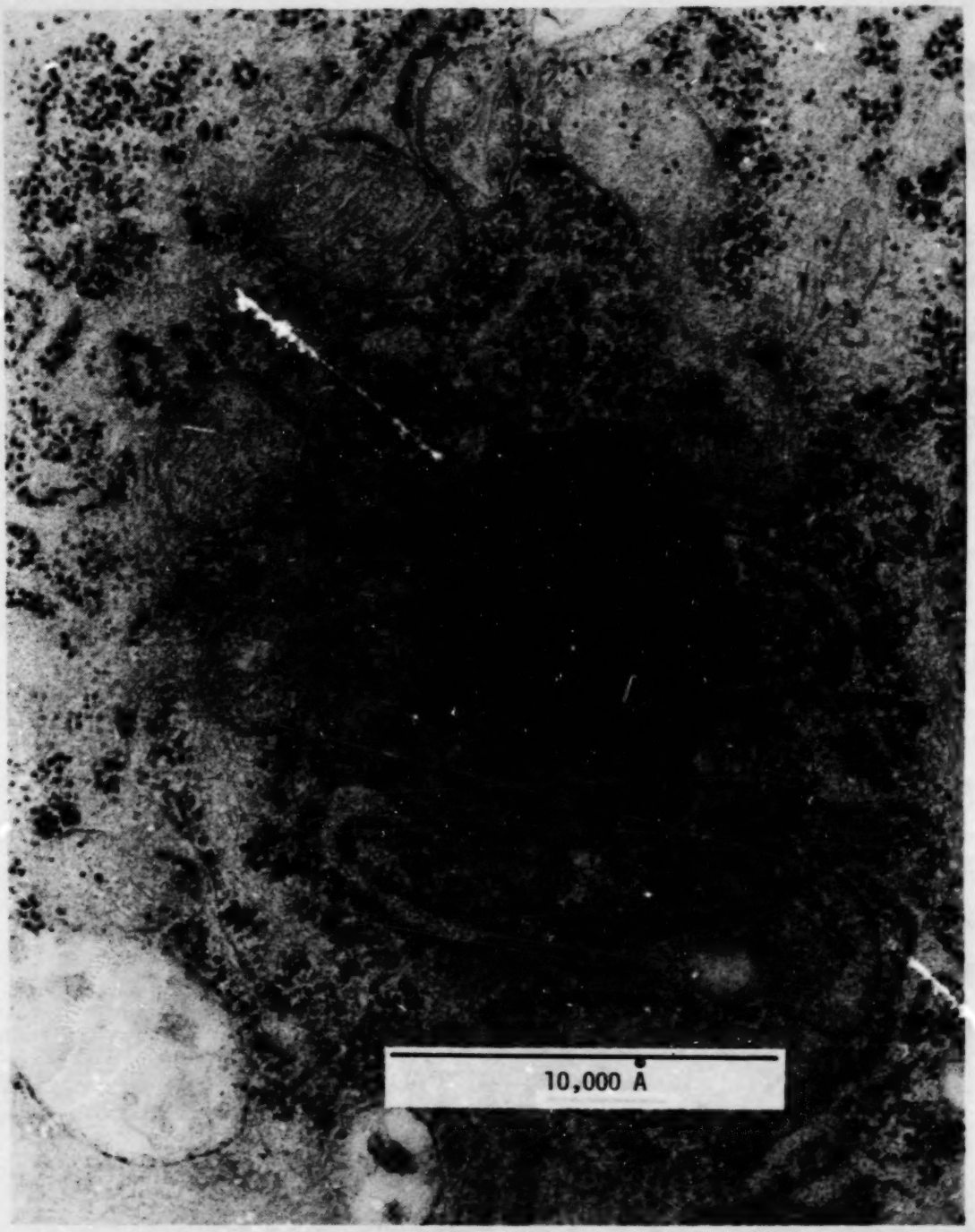


Figure 11. Morphological characteristics typical of polyhedra

Figure 12. Bizarre morphology of monkey cells maintained at 28°C

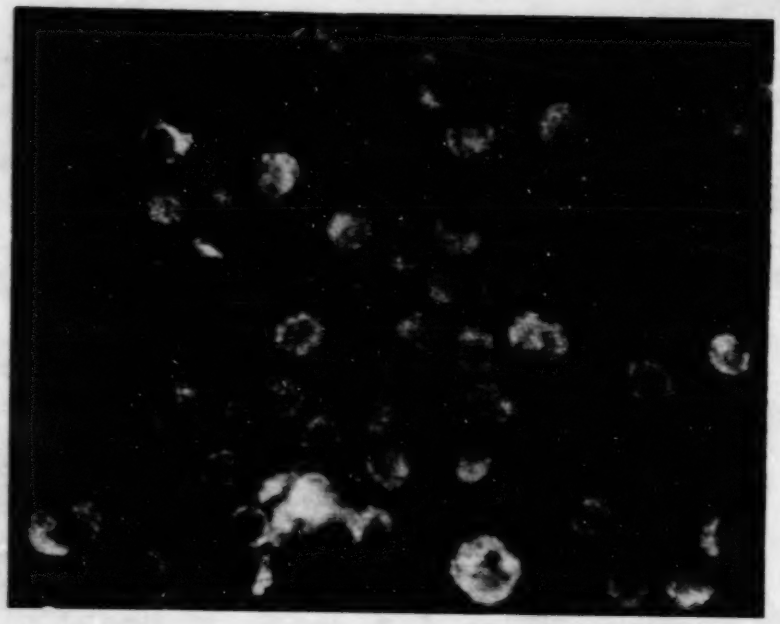


Figure 13. Fluorescence in nuclei of infected T. ni cells

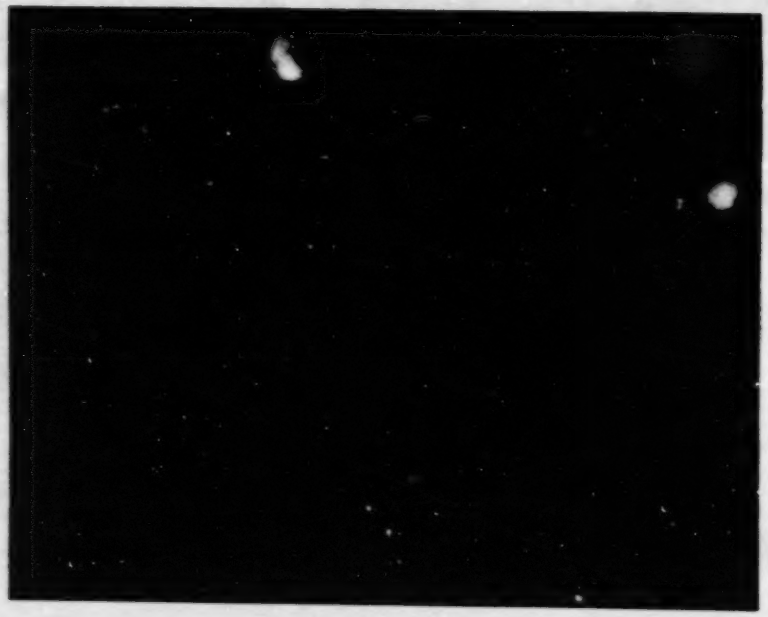


Figure 14. Control for T. ni cell fluorescence experiment

These experiments, however, did not provide any information as to the fate of the infecting virus particles or the viral nucleic acid. Rather, they were designed to test for gross pathologic changes which might be induced by the insect virus.

It was therefore of interest to investigate the possibility that the virus might have adsorbed to the cells, penetrated the cells, and thus able to persist in an "occult" or latent form. This is crucial to the question of whether the virus represents a possible biohazard since in such cases of latent persistence, possible activation of virus has been known to result in an overt pathology.

SECTION 6

TO DETERMINE WHETHER FREE, NON-OCCLUDED AUTOGRAPHICA CALIFORNICA NUCLEAR POLYHEDROSIS VIRUS CAN ADSORB AND PENETRATE MAMMALIAN CELLS IN VITRO

The experiments described below were designed to answer the following questions:

1. Do virus particles of A. californica NPV adsorb and/or penetrate CV-1 monkey cells?
2. Does the viral nucleic acid of A. californica NPV persist in the monkey cells throughout several cell divisions?

Virus was prepared which had a radioactive label incorporated into its nucleic acid. This radioactive virus was used to infect monkey cells. The penetration of the virus into the cells was monitored by determining the quantity of radioactivity taken up by the cells. To ascertain the fate of the infecting nucleic acid, cells were lysed, and the nucleic acid extracted and analyzed by density gradient sedimentation. In the following sections these experiments are described in detail.

PREPARATION OF ^{3}H-LABELED FREE NON-OCCLUDED A. CALIFORNICA VIRIONS IN T. ni CELLS IN VITRO (USED FOR INFECTION OF MONKEY CELLS)

Cell monolayers of T. ni were infected as described previously. The infected cells were overlaid with media containing 50 μCi/mℓ of ^{3}H-thymidine and incubated 48 hours at 28°C. Growth media was removed and centrifuged at 700 x g for 10 minutes at 4°C. The resulting pellet (polyhedra and cell debris) was combined with the cells.

The supernatant (growth media containing infectious free non-occluded NPV) was centrifuged at 27,000 rpm for 60 minutes to concentrate the virions. The virus pellet was resuspended to 1.0 mℓ of Tris-NaCℓ buffer (0.05 M Tris, 0.3 M NaCℓ pH 7.5). Analysis of the concentrated free virions by velocity sedimentation in 10 to 40 percent sucrose gradients is shown in Figure 15. In this figure the free virions are shown to be sedimenting at about the same rate as ^{14}C-labeled T_4 bacteriophage, added here as a marker. (Note: When extrapolated to zero sucrose concentration, T_4 sediments at about 700 S. Since it is known that the A. cal. virions are much less dense than T_4, the A. cal. standard sedimentation rate would be much greater, i.e., about 1000 S.) The peak at the top of the gradient is free DNA. It is not known how much of this DNA represents contamination due to breakdown of cellular DNA.

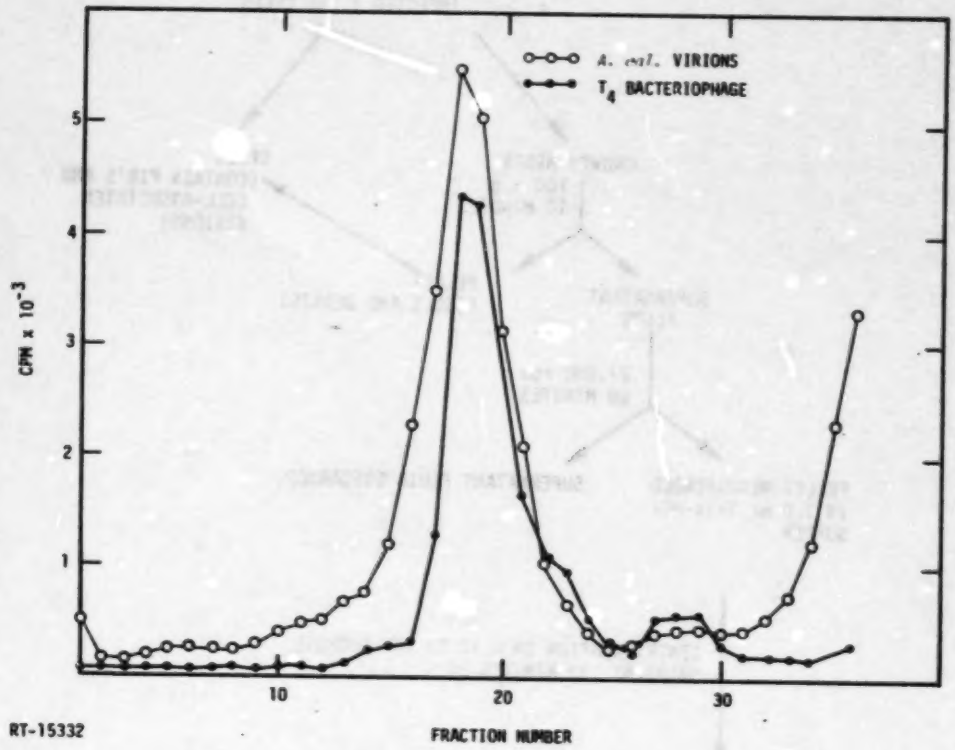


Figure 15. Velocity sedimentation of A. cal. virions in a sucrose gradient. ^{3}H-labeled A. cal. non-occluded NPV was concentrated by sedimentation and resuspended in Tris-NaCℓ buffer, pH 7.5 (see text). An aliquot was removed, mixed with ^{14}C-labeled T_4 bacteriophage (added as a marker) and layered onto a 5 mℓ 10 to 40% (wt/wt) sucrose gradient. The sucrose gradient was centrifuged for 30 minutes at 24,000 rpm at 23°C in an SW50.1 rotor. Fractions were collected and assayed for TCA-precipitable radioactivity.

To purify non-occluded virions for infection of monkey kidney cells, the remainder of the concentrated virion preparation was layered onto a similar 10 to 40 percent sucrose gradient and centrifuged as above (see Figure 16). The fractions containing labeled virions (#14-#22) were pooled and dialyzed into phosphate buffered saline for subsequent infection. The final preparation was characterized by an absorbance spectrum ratio at 260/280 nm of 1.59, and contained about 500,000 cpm.

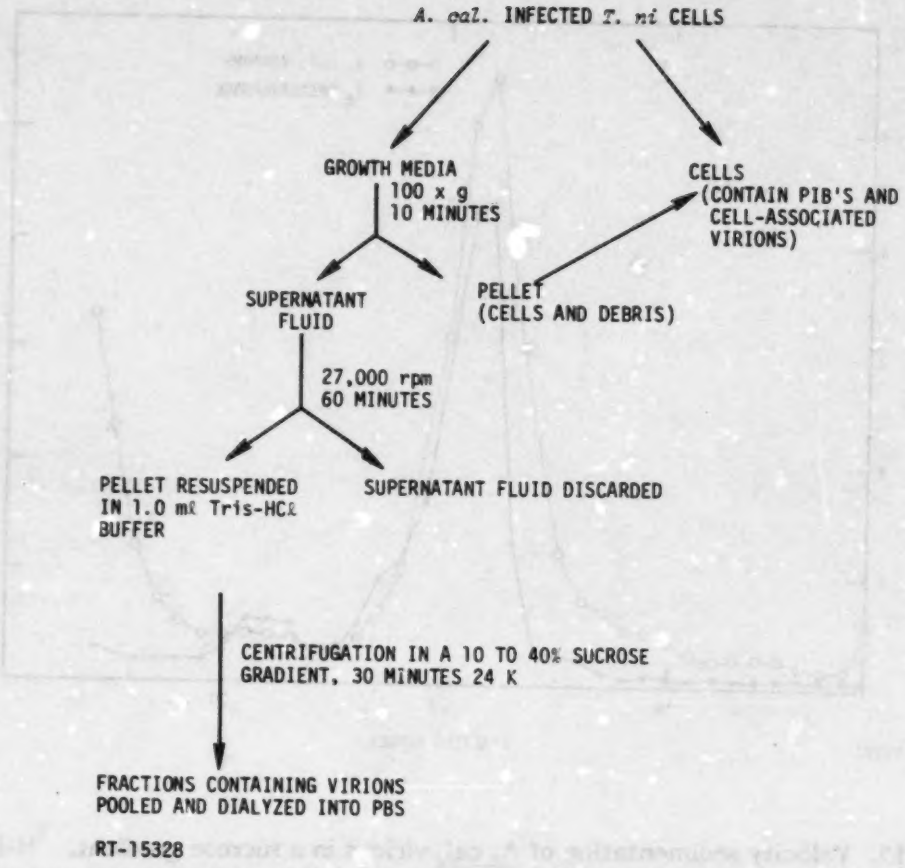


Figure 16. Purification of ^{3}H-labeled A. cal. non-occluded NPV

INFECTION OF MONKEY KIDNEY CELL CULTURES WITH ^{3}H-LABELED A. cal. NON-OCCLUDED VIRIONS

Small petri plate cultures (2.0 cm) containing either CV-1 cells (monkey kidney) or T. ni cells were prepared by mixing 0.2 mℓ of virus in PBS (80,000 cpm) with 0.1 mℓ of Hink's growth media (TNM-FH). Cells were incubated either 2, 4, or 6 hours (CV-1 cells were placed in a 5 percent CO_2 atmosphere). At each of these times, the inocula were withdrawn and counted in a liquid scintillation counter. (Prior to counting, the samples were precipitated with TCA and washed with alcohol to remove any acid soluble material.)

The results are presented in Figure 17. During the first two hours of infection, about 60 percent of the A. cal. virus was absorbed by the T. ni cells. Surprisingly, CV-1 cells adsorbed a similar or even greater percentage of the infecting virus. This is

remarkable when it is considered that no cytopathic effect was detected by conventional methods. Further incubation of CV-1 cells with virus resulted in the adsorption of about an additional 20 percent over the next four hours (Figure 16).

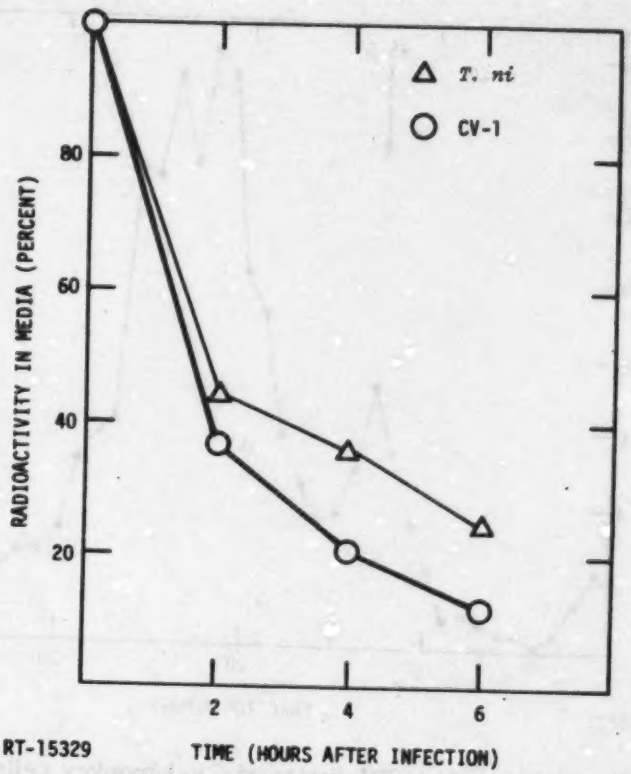


Figure 17. Uptake of radioactive A. cal. virions by CV-1 monkey cells and T. ni insect cells. Purified ^{3}H-labeled A. cal. virions (80,000 cpm/dish) (see text) were added to either CV-1 or T. ni cells and allowed to adsorb at 28°C. At 2, 4, and 6 hours, growth media was removed, centrifuged to remove any unattached cells, and samples of the media were counted in a scintillation counter. The percentage of radioactivity remaining in growth media is plotted against time after infection

In an attempt to determine whether the virus was loosely associated with the cells or bound in a stable association, the cells were washed three times with PBS, then lysed with 0.2 mℓ of 0.6 percent SDS (in 0.01 M EDTA pH 7.5). The resulting lysate was layered onto a 5 mℓ 5 to 20 percent sucrose gradient (w/w) and centrifuged at 40,000 rpm for one hour in an SW56 swinging bucket rotor at room temperature.

The results of this experiment are shown in Figure 18. This figure shows the sedimentation pattern of viral DNA in SDS lysates prepared from CV-1 infected

cultures six hours following infection. Even after six hours, it can be observed that most of the viral DNA sediments as high molecular species. About 20 percent of the DNA is found sedimenting at a rate comparable to intact DNA, while the remainder sediments at rates corresponding to breakdown products. These breakdown products represent molecules that were generated by introducing from 1 to about 9 double-stranded breaks in the native molecule. Interestingly, this type of partial degradation is also found in lysates of infected T. ni cells (data not shown).

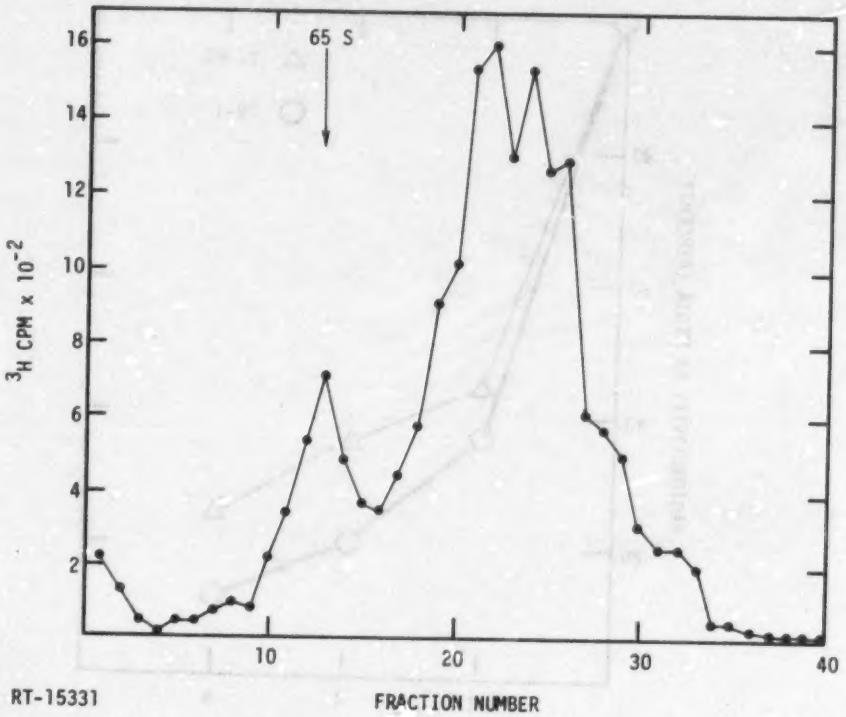


Figure 18. Sedimentation of an SDS lysate of CV-1 monkey cells six hours following infection by ^{3}H-labeled A. cal. virions. Six hours following infection of CV-1 cells with ^{3}H-labeled A. cal. virions (80,000 cpm/dish), cells were washed three times with PBS and 0.2 mℓ of SDS added to each petri dish. The resulting lysate was gently poured from the dish (to avoid shearing the DNA) and the lysate placed onto a 5 mℓ 5 to 20% sucrose gradient. The gradient was centrifuged for one hour at 40,000 rpm in a Spinco SW56 rotor. Centrifugation was from right to left.

In order to determine how much of the total adsorbed DNA this represents, the radioactivity in the gradients was summed and corrected for quench. The results are summarized in Table 2. Lysates prepared from CV-1 cells four hours after infection reveal that at least 79 percent of the adsorbed virus can be accounted for, while after six hours this figure is about 52 percent. Thus, it appears that most of the virus which

adsorbs to the monkey cells remains associated with the cells even after washes. Furthermore, the DNA persists in high molecular weight species. The fact that the DNA remains in a high molecular weight form suggests that this DNA could conceivably be transcribed into viral gene products. However, it should be pointed out that these experiments as performed above are unable to distinguish between adsorbed virus and intracellular virus.

TABLE 2. UPTAKE OF RADIOACTIVE A. cal. VIRIONS BY T. ni CELLS

Cell Type	Time After Infection	Radioactivity[a] Not Adsorbed	Maximum[b] Radioactivity Adsorbed	Radioactivity[c] as Cell-Associated DNA
CV-1	2 hours	29,500	50,500	—
T. ni	2 hours	35,500	44,500	34,588
CV-1	4 hours	16,300	63,700	50,552
T. ni	4 hours	28,600	51,400	—
CV-1	6 hours	10,000	70,000	36,098
T. ni	6 hours	18,800	61,200	—

[a]Radioactivity in growth media removed from cells after infection.

[b]Radioactivity not adsorbed and assumed to be associated with cells. Sampling of washes showed little elution of virus.

[c]Radioactivity in SDS lysates sedimenting as TCA precipitable DNA (see Figure 18).

PERSISTENCE OF A. californica DNA IN INFECTED CV-1 MONKEY CELLS

The experiments presented in the previous section provide evidence for the adsorption and possible entry of A. cal. virions in CV-1 monkey cells. Nonetheless, earlier experiments (see subsection 4.2) failed to reveal a cytopathology in the infected monkey cells.

To determine whether A. cal. viral genes were able to persist in the monkey cells, infected cells were cultured for several passages, and the DNA extracted and concentrated. Also, DNA was extracted from A. cal. PIB's and labeled in vitro using a "nick-repair" method employing E. coli DNA polymerase. The purpose of these procedures was to make labeled viral DNA to use as a probe in DNA-DNA hybridization with cellular DNA. Detection of hybrids could indicate persistence of viral genes in the infected cells. The details of these experiments are presented in the following sections.

Extraction of DNA From Infected Monkey Cells

DNA was extracted and purified from 100 to 200 petri plate cultures of infected or control, uninfected monkey cells. This was done in batches of 50 plates each in the following manner:

1. Cells were lysed on the petri plates by adding 1 mℓ of a solution containing 8 M urea, 1 percent SDS, 10^{-3} M EDTA, 0.24 M sodium phosphate pH 6.8.

2. The lysate was homogenized in a commercial Waring blender 3 x 20 seconds at top speed. Prior to shearing, 2 mℓ of isoamyl alcohol was added per 50 mℓ of lysate to reduce foaming.

3. The lysate was added to a column of hydroxyapatite (HA) (prepared by packing approximately three grams of powdered HA into a 1 x 30 column equilibrated with buffer similar to the lysing buffer, except without SDS). Under these conditions, double-stranded DNA sticks to the column while single-stranded DNA, RNA, and protein are eluted.

   The column is washed with 50 mℓ of a buffer containing 8 M urea, 0.24 sodium phosphate, pH 6.8, then with 100 mℓ of 0.14 M sodium phosphate buffer pH 6.8. The absorbancy at 260 nm is monitored, and if the A_{260} is less than 0.1, the DNA is eluted. If it is not below 0.1, additional 0.14 M phosphate buffer is added.

   DNA is eluted by adding 0.48 M phosphate buffer, pH 6.8, and is collected in 3 mℓ aliquots. In a typical purification, the DNA elutes in eight 3 mℓ fractions. DNA eluted from this column is generally contaminated with protein, as indicated by a 260/180 nm ratio less than 1.8. The DNA is therefore treated with pronase (100 μg/mℓ for one hour at 37°C), and extracted with an equal volume of phenol (saturated with 0.2 M Tris, pH 7.5 buffer). The aqueous phase is diluted with water to give a final phosphate concentration of 0.14 M, and then added to a second HA column maintained at 60°C. This column is washed with 100 to 200 mℓ of 0.14 M phosphate buffer, and the DNA eluted with 0.48 M phosphate buffer as described earlier. DNA from this second column is generally clean, as indicated by a 260/280 nm ratio of about 1.85 and by protein and RNA analysis of the DNA.

4. DNA is then sheared to a piece size of about 400 to 600 nucleotides. This is done in a Sorvall Ribi cell fractionator at a force of 50,000 pounds per square inch at 4°C. After shearing, the DNA is concentrated and purified again by passage through an HA column at 60°C.

5. The DNA is then dialyzed extensively against 5×10^{-5} M EDTA for three days. Following dialysis, the DNA is lyophilized and stored at -20°C.

Extraction of DNA from T. ni Cells

DNA was extracted from A. cal. infected and control, uninfected T. ni cells by a procedure similar to that described above for CV-1 cells. Generally, cell DNA was relatively free of contaminating protein after a single passage through an HA column (8 M urea, 1 percent SDS, 0.24 M phosphate buffer used to lyse the cells). Thus, the pronase treatment and phenol extraction steps were omitted and the DNA was simply passed through a second HA column at 60°C, sheared in the cell fractionator, concentrated on an HA column, dialyzed and lyophilized.

Extraction of DNA from Heliothis zea Cells

A Heliothis zea cell line obtained from Dr. Timothy Kurtti was routinely propagated in the laboratory. DNA was extracted from these cells to be used as a

heterologous DNA control in hybridization experiments. The extraction procedure used was similar to that described above for T. ni cell DNA.

Calf Thymus DNA

Calf thymus DNA was obtained commercially from the Sigma Chemical Company. However, it was found to be highly contaminated with protein and RNA. Therefore, this DNA had to be further purified. Commercial, lyophilized calf thymus DNA was hydrated for two days in phosphate buffer (0.14 M, pH 6.8). Thereafter, it was sheared in a Waring blender, treated with pronase, RNase, and purified by passage through an HA column at 60°C. Further purification steps were as described for the DNA's above.

DNA Purity

To test the purities of the DNA's prepared above, each was thermally denatured in a Gilford spectrophotometer equipped with automatic accessories for thermal denaturation studies. In each case, a sample of the DNA was removed before the shearing step, dialyzed extensively against 0.15 M NaCℓ, 0.015 M sodium citrate buffer pH 7, and denatured by heating to 100°C at increments of 0.5 degrees per minute between 50 and 100°C.

Table 3 summarizes the properties of the cellular DNA's prepared for the hybridization studies.

TABLE 3. PROPERTIES OF CELLULAR DNA PREPARED FROM A. cal. NPV INFECTED AND UNINFECTED CELLS

Source of DNA	A260/A280	Total Amount (mg)	Tm	Hyperchromicity (%)
T. ni	1.90	1.52	84.8	31.2
H. zea	2.03	1.97	85.2	33.7
CV-1 Uninfected	1.81	0.64	87.0	32.1

PREPARATION OF IN VITRO LABELED A. cal. NPV DNA

Purification of A. cal. NPV PIB's from A. cal. Larvae

Initial attempts to obtain NPV DNA from infected tissue culture T. ni cells for use as a substrate for E. coli DNA polymerase were unsuccessful for a number of reasons. First and foremost was the fact that a very large amount of infected culture cells yielded only a very small amount of DNA. Secondly, attempts to separate PIB's from cellular components resulted in a loss of yield of PIB's. Therefore, infected A. cal. larvae were used as a source of PIB's, which in turn were extracted to obtain DNA. The infected larvae were supplied to us by Dr. Vail of the USDA, Phoenix, Arizona. The growth of these larvae took several weeks, and, in all, several thousand larvae were provided as a dried powder at 4.64×10^9 PIB's/gram.

For purification of A. cal. PIB's, four grams of powder were weighed out and hydrated in 100 mℓ of 0.05 M KCℓ, 0.05 M Tris buffer pH 7.5. After stirring overnight at 4°C, the PIB's were washed four times by centrifugation at 8,000 rpm for 30 minutes in a Beckman J21 centrifuge and resuspension of the PIB pellet in 50 mℓ of buffer. Washing was stopped when the supernatant was clear and free of visible debris. The washed PIB's were then placed on sucrose gradients formed in steps in centrifuge tubes by the following procedure:

15 mℓ 2.2 M sucrose

10 mℓ 1.625 M sucrose

10 mℓ 1.0 M sucrose

10 mℓ PIB

These gradients were centrifuged to equilibrium at 11,000 rpm in the JS-13 rotor for 120 minutes.

Three thick, opalescent bands were obtained, one at the interface between each of the sucrose density steps. The band between the 2.2 and 1.625 M sucrose contained mostly PIB's, whereas the top two bands (the one between 1.625 and 1.0 M sucrose and the one between 1.0 M sucrose and buffer) contained mostly debris. The bottom PIB band was collected, diluted in distilled water (1:3), centrifuged to concentrate the PIB's, and the equilibrium centrifugation step repeated three more times. After the second equilibrium, only a bottom PIB band was found. The top two bands were combined, concentrated, and treated with 1 percent SDS at 50°C for one hour. Subsequently, the remaining PIB's were concentrated and centrifuged to equilibrium several times, as described above.

Extraction of A. cal. DNA from Purified PIB's

A number of different methods were attempted for the extraction of native high-molecular-weight DNA from A. cal. PIB's. The first method is based on the procedure of Gafford and Randall as modified by Summers and Anderson (Ref 18). Purified polyhedral bodies were made alkaline in the presence of sodium lauryl sarcosine (0.05 M Na_2CO_3, 0.02 M NaCℓ, 10^{-3} M Tris, 10^{-4} M EDTA, 2 percent SLS). The virus solution was incubated at 60°C for 30 minutes. After cooling to room temperature, the solution was titrated to neutrality with 1.7 N acetic acid. The solution was then extracted four times with chloroform:butanol (3:1). During this extraction, as well as during all other procedures, the DNA was handled very gently to avoid breaking the DNA by shear. The aqueous phase was removed and two volumes of alcohol added. The DNA was spooled from solution on a glass rod at the interface between the aqueous and alcohol. The DNA was then dissolved in 0.015 M NaCℓ, 0.-015 M sodium citrate overnight. The 260/280 ratio of this DNA was 1.87. This DNA was subsequently phenol-extracted, but the 260/280 ratio did not increase.

The second method used was found to be simpler and yielded high molecular-weight DNA. This is the method adopted for routine extraction of A. cal. NPV DNA. Purified polyhedral bodies were mixed with an equal volume of 0.2 M Na_3PO_4, pH 10.5 for 20 minutes, by which time the reaction mixture had cleared. SDS was added to a final concentration of 0.2 percent and the solution incubated at 50°C for 30 minutes.

Thereafter, the lysed virus was placed onto a 5 to 20 percent (wt/wt) sucrose gradient and centrifuged at 40,000 rpm for 60 minutes at 23°C in an SW56 rotor. The resultant gradient showing ^{3}H-labeled A. cal. DNA with ^{14}C-labeled SV40 DNA as a marker is shown in Figure 19. The resulting peak of A. cal. DNA is monodispersed and sediments at about 50 S. This DNA peak was pooled and dialyzed against 0.01 M Tris, 10^{-4} EDTA pH 7.5. For preparing DNA for hybridization, a similar procedure was followed using PIB's obtained from larvae (see above), and this DNA was used as a template for E. coli polymerase in the synthesis of a labeled probe. In sucrose gradients, the DNA peak was monitored by determining the absorbance at 260 nm.

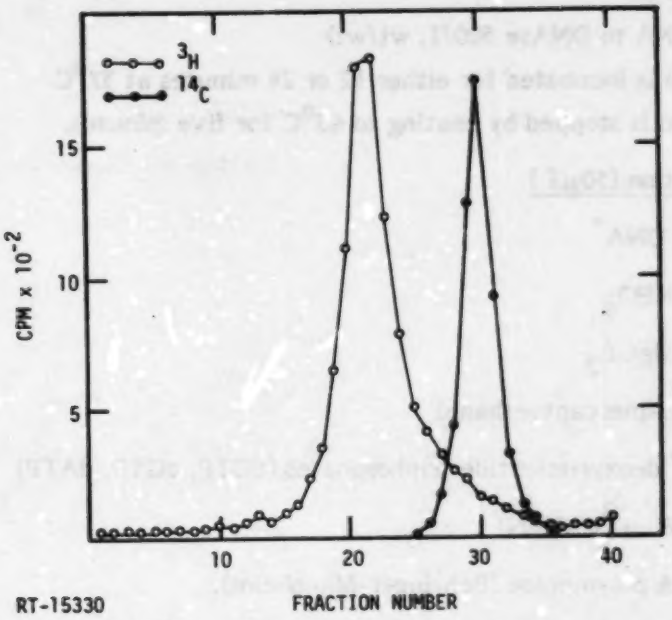


Figure 19. Sedimentation of A. cal. NPV DNA extracted by alkali and SDS from purified PIB. ^{3}H-labeled A. cal. PIB purified from infected T. ni cells was extracted by treatment with SDS at pH 10.5, as described in the text. The polyhedral lysate was mixed with ^{14}C-labeled SV40 DNA added as a marker and sedimented at 40,000 rpm for 60 minutes at 23°C in a Spinco SW56 rotor. Radioactivity represents TCA precipitable counts.

IN VITRO LABELING OF A. cal. NPV DNA EMPLOYING E. coli DNA POLYMERASE

The following method for the in vitro labeling of DNA is a modification of the method first described by Nonoyama and coworkers (Ref 19) and modified by Drs. Wayne Lancaster and William Meinke of Scripps Clinic. High molecular weight, native, double-stranded DNA is reacted with low concentrations of deoxyribonuclease to introduce single-stranded nicks into the DNA. This first reaction is stopped, and the nicked DNA is then reacted with E. coli DNA polymerase under conditions during which

the enzyme simultaneously digests the DNA (5'-end) and then synthesizes complementary base sequences from the 3'-end (translation reaction). By employing radioactively labeled deoxyribonucleotide triphosphate precursors, the labeled bases are incorporated in short stretches. In this way, labeled DNA can be obtained with specific activities of several million cpm/μg of DNA. A typical experiment is described as follows.

Nicking Reaction (0.642 mℓ)

5 μg/mℓ DNA in

0.03 M Tris, pH 7.5

0.03 M KCℓ

0.003 M $MgC\ell_2$

0.01 μg DNAse

(Ratio of DNA to DNAse 500/1, wt/wt)

The mixture is incubated for either 12 or 24 minutes at 37°C

The reaction is stopped by heating to 65°C for five minutes.

Translation Reaction (50 μℓ)

5×10^{-2} μg DNA*

5×10^{-2} M KPO_4

5×10^{-3} M $MgC\ell_2$

5×10^{-3} M β-mercaptoethanol

$3 \times 5 \times 10^{-5}$ deoxynucleotide triphosphates (dCTP, dGTP, dATP)

10 μCi dTTP → ^{3}H (NEN)

5 μℓ of DNA polymerase (Bohringer-Mannheim).

Mixture was incubated for 30 minutes at 20°C.

Reaction stopped by heating at 68°C for five minutes.

Purification of the Labeled DNA

In order to remove the unreacted bases and label, the reaction mixture was passed over a G50 Sephadex column equilibrated with 0.02 M Tris, pH 8, 10^{-3} M EDTA and 0.1 percent Sarkosyl; 50 μℓ was applied to a column, 0.7 x 22 cm. The labeled DNA was eluted in a void volume, while the low molecular material was retarded. The labeled DNA was pooled and counted in a liquid scintillation counter. The size of the DNA was determined by alkali sucrose gradient centrifugation. The reason for this latter

*Reactions carried out using DNA that was nicked for either 12 or 24 minutes (see above).

procedure was that there is an optimum piece size for reassociation, and this is between 200 and 600 nucleotide base pairs. Thus, the analysis by alkaline sucrose density gradient centrifugation was a monitor of the extent of the nicking reaction, as well as a test of the extent of nuclease versus polymerase in the translation reaction.

In the alkaline gradient, a 7 S ^{14}C-labeled human cellular DNA was used as a marker. The results of this nick-translation are shown in Table 4. With both a 12- and 24-minute incubation, the amount of incorporation into A. cal. was about 1×10^6 cpm/μg. This is exceedingly high compared to in vivo labeling; however, it is only about 15 percent of the optimum amount of incorporation usually obtained by this method using small molecular-weight viral DNA's such as SV40 DNA or human papilloma DNA. Furthermore, the size of the DNA was found to be too large for optimum hybridization. This would suggest that the nicking times and/or deoxyribonuclease concentrations must be changed. Experiments are presently in progress to improve the efficiency of incorporation, as well as to adjust reaction conditions in order to generate a labeled product with the properties of a suitable probe.

TABLE 4. NICK-TRANSLATION OF A. cal. DNA BY E. coli DNA POLYMERASE

DNase Treatment Time (minutes)	Specific Activity of DNA (cpm/μg)	Sedimentation Rate of DNA in Alkali S (Svedbergs)
12	1,200,000	>10
24	1,000,000	>10

SECTION 7

THE USE OF DNA-DNA HYBRIDIZATION FOR DETECTION OF THE PERSISTENCE OF VIRAL GENOMES

ISOLATION OF VIRAL DNA

Characterization of In Vivo Labeled DNA

A. cal. NPV was grown in T. ni cells in the presence of ^{3}H-thymidine, and virions were isolated from the supernatant medium by centrifugation through linear 5-40 percent potassium tartrate gradients. Radioactively labeled viral DNA was extracted from the purified virions by treatment with SDS and phenol. Equilibrium centrifugation of the viral DNA preparation through cesium chloride and ethidium bromide allowed the separation of supercoiled viral DNA and open circular and linear DNA forms (Figure 20).

Preparation of Unlabeled Viral DNA

Unlabeled viral DNA for use in the in vitro labeling procedure was extracted directly from preparations of dried, frozen A. cal. infected larvae. Two to three grams of the larval preparation were resuspended in 6 mℓ of distilled water and brought to 0.03 M $NaCO_3$ (pH 10.9). This solution was incubated at 50°C for ten minutes then made to 0.01 M EDTA and 1 percent sodium lauryl sarcosinate, and incubated at 50°C for an additional 15 minutes. Heavy debris was removed by low speed centrifugation, and the supernatant was layered directly onto preformed CsCℓ/ethidium bromide gradients (ρ 1.48-1.60 in 0.01 M Tris, pH 7.5, 1 mM EDTA, 200 μg/mℓ ethidium bromide) and centrifuged at 100,000 x g for 18 hours. DNA in the preparation bound ethidium bromide, and was visible as a thick red band near the bottom of the centrifuge tube. This band and any pelleted material were resuspended in 2.5 mℓ of the above buffer, and the solution was brought to ρ 1.602 by addition of CsCℓ. The solution was covered with mineral oil and centrifuged at 32,000 rpm for 48 hours at 25°C in the SW50.1 rotor. Supercoiled viral DNA and open circular and linear DNA were visible as two distinct bands under UV light. Only supercoiled viral DNA from the bottom band was utilized in preparing in vitro labeled DNA. This ensured that only viral DNA, and no residual cellular DNA, would be included in the labeled probe.

PREPARATION OF IN VITRO LABELED VIRAL DNA

Viral DNA was labeled in vitro by the "nick-repair" technique of Lancaster and Meinke (Refs 15,16) (Figure 21). This procedure allows the preparation of labeled DNA with specific activities of 10^{6}-10^{7} cpm/μg of DNA, higher than can be achieved through in vivo labeling. This method has the added advantage of requiring only a small amount of viral DNA (1 μg) for each reaction.

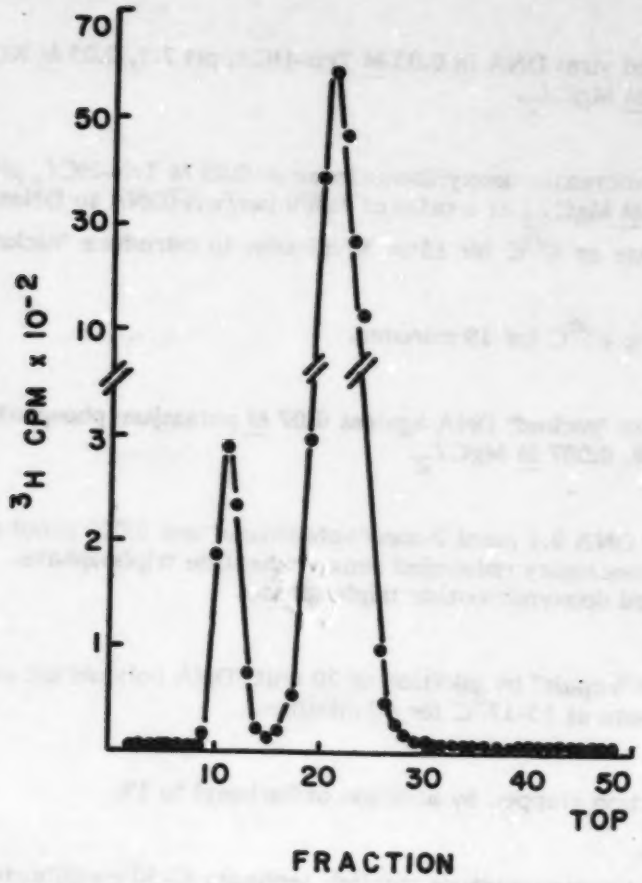


Figure 20. CsCℓ-dye equilibrium centrifugation of A. cal. viral DNA. CsCℓ(1 g/mℓ) was dissolved in DNA solutions and ethidium bromide added to a final concentration of 100 mg/mℓ in a volume of 3.0 mℓ. The density of the solution was adjusted to 1.5786, the solution covered with mineral oil and centrifuged at 32,000 rpm for 48 hours at 25°C in the 50.1 rotor. Fractions were collected from the bottom of the tube directly onto filter paper discs and assayed for radioactivity following treatment with trichloroacetic acid and washing with 95% ethanol. The heavy peak (a) corresponds to closed supercoiled viral DNA while the light peak (b) represents open circular and linear form.

A. cal. supercoiled DNA was treated with pancreatic deoxyribonuclease I at a ratio of 1:100 (DNA:DNase) to introduce single-stranded scissions. The nuclease was inactivated by heating to 65°C for 19 minutes. Viral DNA was subsequently labeled by initiating repair synthesis with the addition of Escherichia coli DNA polymerase I in the presence of unlabeled ATP, CTP, and GTP and ^{3}H-TTP. The reaction was held at 15-17°C to reduce the possibility of displacement synthesis. Repair synthesis was terminated by the addition of Sarkosyl, and the DNA was separated from unincorporated nucleotides on a Sephadex G-50 column. This DNA preparation has a specific activity of 7.5×10^6 cpm/μg DNA.

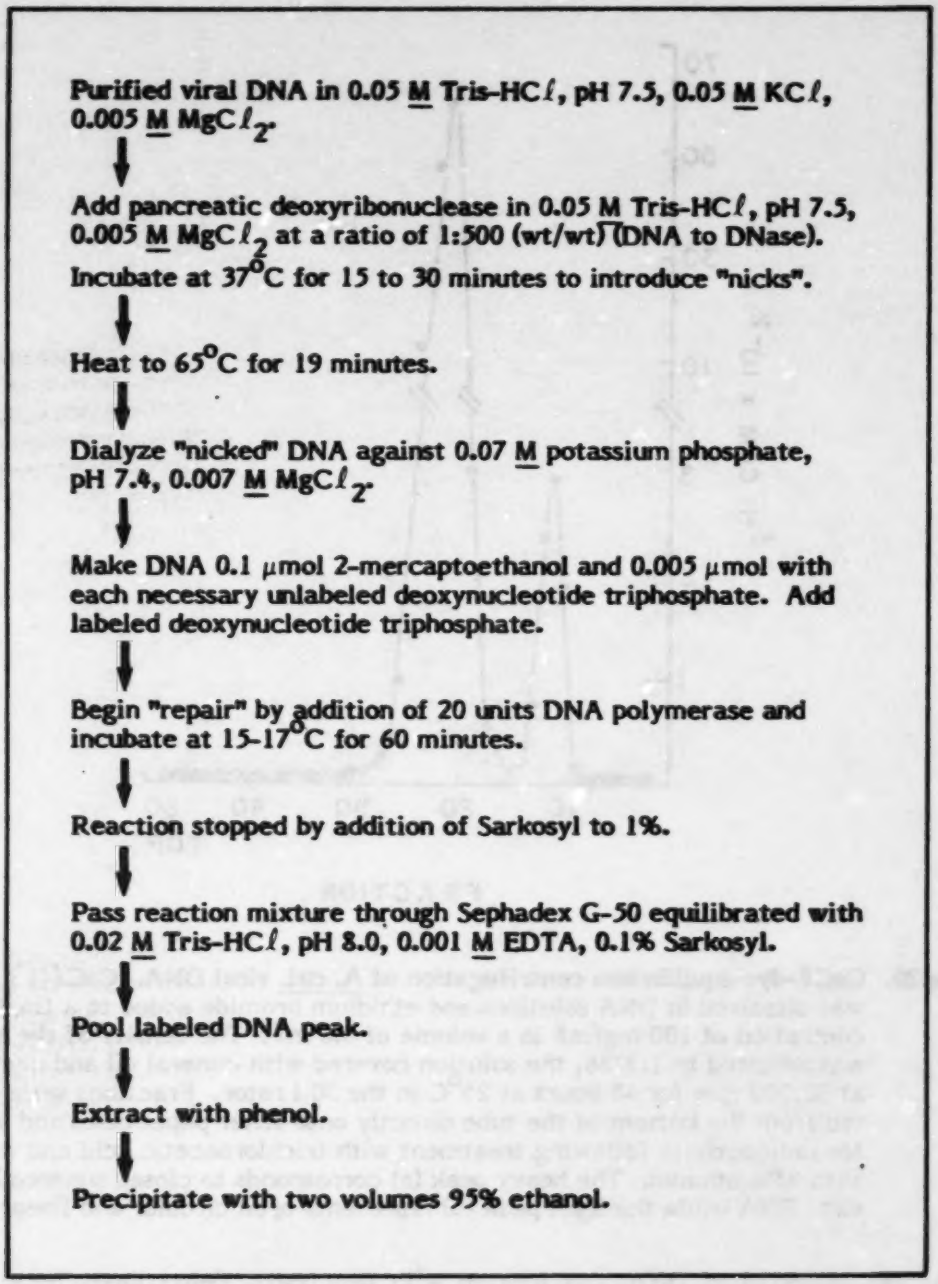

Purified viral DNA in 0.05 M Tris-HCℓ, pH 7.5, 0.05 M KCℓ, 0.005 M MgCℓ2.
Add pancreatic deoxyribonuclease in 0.05 M Tris-HCℓ, pH 7.5, 0.005 M MgCℓ2 at a ratio of 1:500 (wt/wt) (DNA to DNase).
Incubate at 37°C for 15 to 30 minutes to introduce "nicks".
Heat to 65°C for 19 minutes.
Dialyze "nicked" DNA against 0.07 M potassium phosphate, pH 7.4, 0.007 M MgCℓ2.
Make DNA 0.1 μmol 2-mercaptoethanol and 0.005 μmol with each necessary unlabeled deoxynucleotide triphosphate. Add labeled deoxynucleotide triphosphate.
Begin "repair" by addition of 20 units DNA polymerase and incubate at 15-17°C for 60 minutes.
Reaction stopped by addition of Sarkosyl to 1%.
Pass reaction mixture through Sephadex G-50 equilibrated with 0.02 M Tris-HCℓ, pH 8.0, 0.001 M EDTA, 0.1% Sarkosyl.
Pool labeled DNA peak.
Extract with phenol.
Precipitate with two volumes 95% ethanol.

Figure 21. In vitro labeling of viral DNA

The in vitro labeled A. cal. DNA was sized by sedimentation through a 5-20 percent alkaline sucrose gradient at 49,000 rpm for seven hours at 15°C in the SW50.1 rotor (Figure 22). The DNA sedimented at about 7.5S, when compared with a 7S 14C-WiL2 DNA marker.

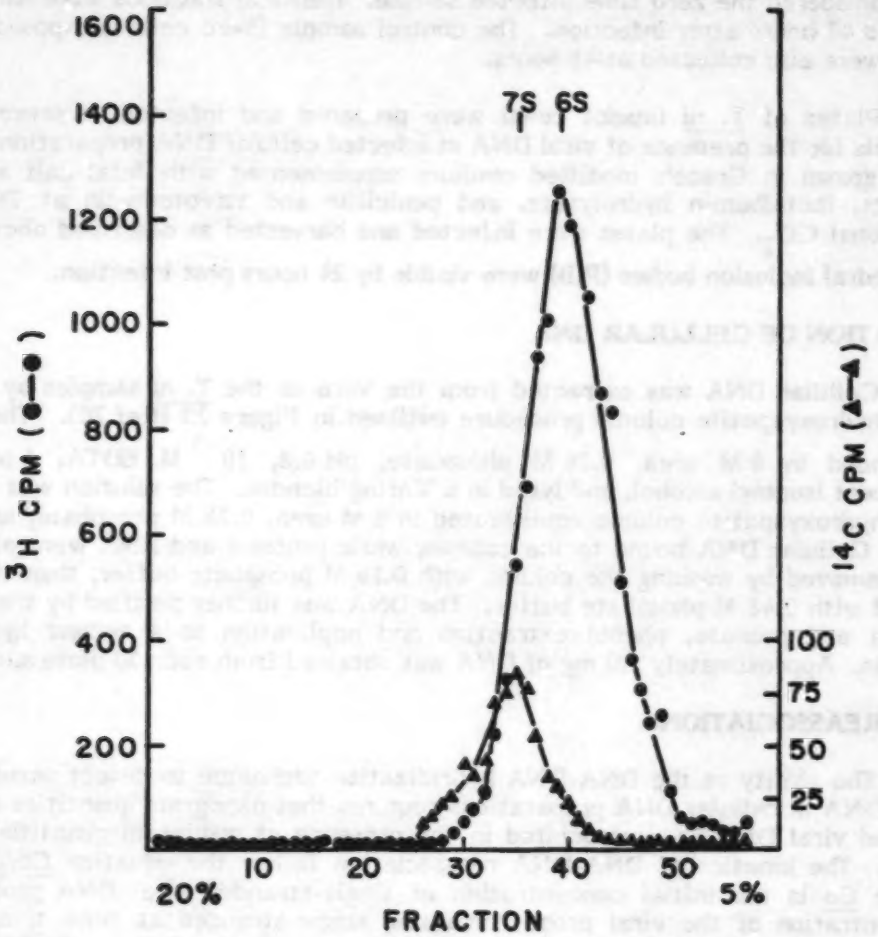


Figure 22. Alkaline sucrose gradient centrifugation of in vitro labeled A. cal. DNA. DNA samples were centrifuged through 5-10% linear sucrose gradients in 0.3 N NaOH, 1.0 M NaCℓ, 10-3 M EDTA and 0.015% Sarkosyl in the SW56 rotor at 49,000 rpm for seven hours at 15°C. 14C WiL2 DNA (7S) was added as a marker. Fractions were collected from the bottom of the tube onto filter paper discs and assayed for radioactivity following treatment with trichloroacetic acid.

INFECTION OF CELL LINES

Vero (African green monkey kidney) cells were grown in Eagle's medium supplemented with 10 percent fetal calf serum and penicillin and streptomycin at 37°C in a 5 percent CO_2 atmosphere. Two-hundred fifty plastic plates (100 cm) of cells were prepared, and 200 plates were infected when the monolayers were approximately 80 percent confluent. An inoculum of 5×10^6 plaque forming units (pfu) per plate were used for a multiplicity of infection of approximately one. The virus was allowed to absorb at 37°C for 90 hours, then the cells from 50 plates were removed by trypsinization, pelleted by low speed centrifugation, and frozen at -20°C. This sample was considered the zero time infected sample. Identical fractions were harvested at 4, 24, and 48 hours after infection. The control sample (Vero cells unexposed to A. cal. NPV) were also collected at 48 hours.

Plates of T. ni (insect cells) were prepared and infected to serve as positive controls for the presence of viral DNA in infected cellular DNA preparations. The cells were grown in Grace's modified medium supplemented with fetal calf serum, yeast extract, lactalbumin hydrolysate, and penicillin and streptomycin at 26°C without additional CO_2. The plates were infected and harvested as described above. Nuclear polyhedral inclusion bodies (PIB) were visible by 24 hours post infection.

ISOLATION OF CELLULAR DNA

Cellular DNA was extracted from the Vero or the T. ni samples by a ureaphosphatehydroxyapatite column procedure outlined in Figure 23 (Ref 20). The cells were suspended in 8 M urea, 0.24 M phosphate, pH 6.8, 10^{-3} M EDTA, 1 percent SDS, 2 percent isoamyl alcohol, and lysed in a Waring blender. The solution was then applied to a hydroxyapatite column equilibrated in 8 M urea, 0.24 M phosphate, and 1 percent SDS. Cellular DNA bound to the column, while proteins and RNA were eluted. Urea was removed by washing the column with 0.14 M phosphate buffer, then the DNA was eluted with 0.48 M phosphate buffer. The DNA was further purified by treatment with RNase and pronase, phenol extraction and application to a second hydroxyapatite column. Approximately 1.0 mg of DNA was obtained from each 50 plate sample.

DNA REASSOCIATION

The ability of the DNA-DNA hybridization technique to detect small amounts of viral DNA in cellular DNA preparations requires that nanogram quantities of denatured labeled viral DNA be reassociated in the presence of milligram quantities of cellular DNA. The kinetics of DNA-DNA reassociation follow the equation $Co/C = 1 + KCot$ where Co is the initial concentration of single-stranded viral DNA probe, C is the concentration of the viral probe remaining single-stranded at time t, and K is the reassociation constant. A plot of Co/C vertus t will result in a straight line if the DNA-DNA reassociation reactions are following second-order kinetics. If unlabeled viral DNA sequences are present in DNA extracted from infected cells, the reassociation rate of the probe will be increased in direct proportion to the amount of unlabeled viral DNA present. The total amount of viral DNA present in a sample of infected cellular DNA can be determined by the increase in the reassociation rate.

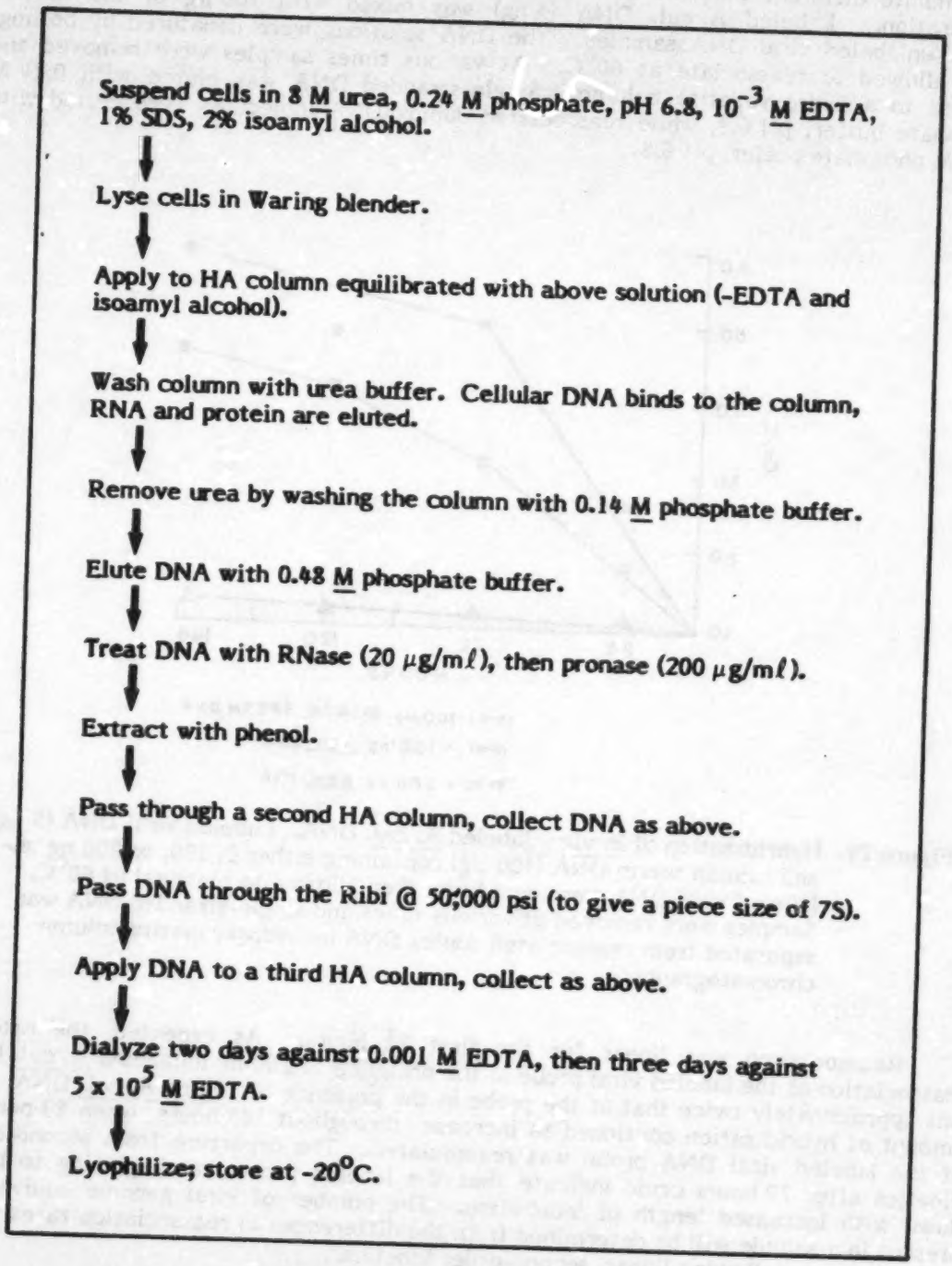

Suspend cells in 8 M urea, 0.24 M phosphate, pH 6.8, 10^{-3} M EDTA, 1% SDS, 2% isoamyl alcohol.
Lyse cells in Waring blender.
Apply to HA column equilibrated with above solution (-EDTA and isoamyl alcohol).
Wash column with urea buffer. Cellular DNA binds to the column, RNA and protein are eluted.
Remove urea by washing the column with 0.14 M phosphate buffer.
Elute DNA with 0.48 M phosphate buffer.
Treat DNA with RNase (20 μg/mℓ), then pronase (200 μg/mℓ).
Extract with phenol.
Pass through a second HA column, collect DNA as above.
Pass DNA through the Ribi @ 50,000 psi (to give a piece size of 7S).
Apply DNA to a third HA column, collect as above.
Dialyze two days against 0.001 M EDTA, then three days against 5×10^{5} M EDTA.
Lyophilize; store at -20°C.

Figure 23. Schematic of extraction of cellular DNA

Figure 24 illustrates data from a DNA-DNA reassociation experiment set up to determine the effectiveness of the in vitro labeled A. cal. DNA probe. Salmon sperm DNA (100 μg) was mixed with varying concentrations of purified unlabeled A. cal. DNA to simulate different amounts of unlabeled viral DNA in an infected cellular DNA preparation. Labeled A. cal. DNA (5 ng) was mixed with 100 μg of the salmon sperm/unlabeled viral DNA samples. The DNA solutions were denatured by boiling, then allowed to reassociate at 60°C. At various times samples were removed and applied to a hydroxyapatite column. Single-stranded DNA was eluted with 0.14 M phosphate buffer, pH 6.8, while reassociated duplex DNA molecules were eluted with 0.48 M phosphate buffer, pH 6.8.

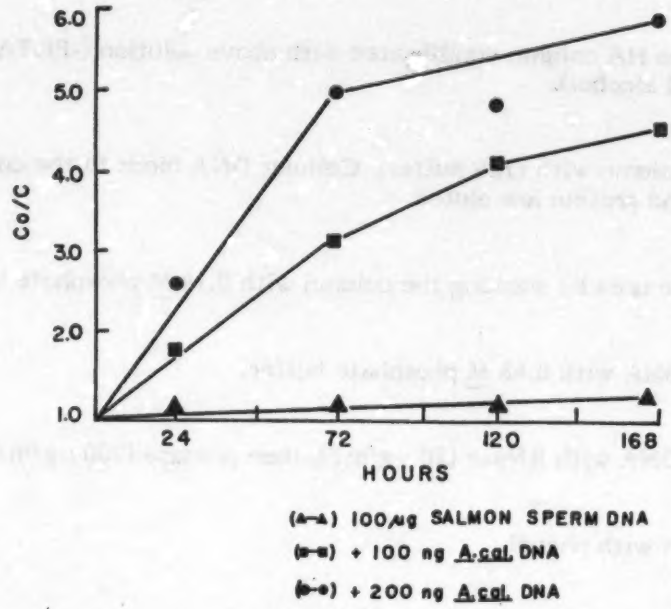


Figure 24. Hybridization of in vitro labeled A. cal. DNA. Labeled viral DNA (5 ng) and salmon sperm DNA (100 μg) containing either 0, 100, or 200 ng unlabeled viral DNA were denatured then allowed to reanneal at 60°C. Samples were removed at various times and single-stranded DNA was separated from reassociated duplex DNA by hydroxyapatite column chromatography.

Reassociation was linear for the first 72 hours. As expected, the rate of reassociation of the labeled viral probe in the presence of 200 ng unlabeled A. cal. DNA was approximately twice that of the probe in the presence of 100 ng A. cal. DNA. The amount of hybridization continued to increase throughout 168 hours, when 83 percent of the labeled viral DNA probe was reassociated. The departure from second-order kinetics after 72 hours could indicate that the labeled probe was beginning to break down with increased length of incubation. The number of viral genome equivalents present in a sample will be determined from the differences in reassociation rates while the reaction is following linear, second-order kinetics.

SECTION 8

SUMMARY

- Large quantities of cellular DNA have been extracted from various control and virus infected cultures. This DNA has been characterized, sized, and lyophilized.
- Large quantities of NPV virus have been purified and the DNA extracted.
- The feasibility of preparing in vitro labeled A. cal. DNA and utilizing this as a probe to follow the fate of insect virus genomes in infected cells has been established.
- It has been shown that DNA labeled in vitro will hybridize with unlabeled A. cal. DNA in a mixed DNA preparation and is thus a sensitive probe for the detection of viral DNA.

SECTION 9

REFERENCES

1. P. Collins, Nature 257:2-3 (1975).

2. R. F. Smith, "Baculoviruses for Insect Pest Control: Safety Considerations," American Society for Microbiology, pp. 9-11.

3. WHO Technical Report Series No. 531 (FAO Agricultural Studies, No. 91), Report of the Joint FAO/WHO Meeting on Insect Viruses, Geneve (1972) (World Health Organization, Geneve, 1973).

4. M. H. Rogoff, "Baculoviruses for Insect Pest Control," American Society for Microbiology, pp. 102-103.

5. C. M. Ignoffo, Ann. Y. Y. Acad. Sci. 217:141-164 (1973).

6. R. Rubinstein and A. Polson, J. Insect. Path. 28:157-160 (1976).

7. H. L. Morton, J. O. Moffett, and F. D. Stewart, J. Insect. Path. 26:139-140 (1975).

8. A. H. McIntosh, "Baculoviruses for Insect Pest Control: Safety Considerations," American Society for Microbiology, pp. 63-69 (1975).

9. R. H. Goodwin, J. L. Vaughn, J. R. Adams, and S. J. Louloudes, Pub. Ent. Soc. Am. 9:66-72 (1973).

10. M. Himeno, F. Saki, K. Omodera, H. Nakai, T. Fukada, Y. Kawade, Virology 33:507-512 (1967).

11. C. M. Ignoffo and R. R. Rafajko, J. Invertbr. Path. 20:321-325 (1972).

12. A. H. McIntosh and K. Maramorosch, J. N. Y. Entomol. Soc. 81:175-182 (1973).

13. C. M. Ignoffo, M. Shapiro, and W. F. Hink, J. Invert. Path. 18:131-134 (1971).

14. K. N. Potter, P. Faulkner, and E. A. MacKinnon, J. Virol. 18:1040-1050 (1976).

15. W. D. Lancaster and W. Meinke, Nature (London) 256:434-436 (1975).

16. W. D. Lancaster, C. Olson, and W. Meinke, J. Virol 17:824-831 (1976).

17. L. D. Gelb, D. E. Kohne, and M. A. Martin, J. Mol. Biol. 57:129-145 (1971).

18. M. D. Sumners and D. L. Anderson, Virology 50, pp. 459-471 (1972).

19. M. Nonoyama and J. S. Pagano, Nature 242, pp. 44-47 (1973).

20. W. Meinke, D. A. Goldstein, and M. R. Hall, Anal. Biochem. 58:82-88 (1974).

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/1-80-026	2.	3. RECIPIENT'S ACCESSION NO. PB80 202427
4. TITLE AND SUBTITLE Investigation of the Persistence and Replication of Nuclear Polyhedrosis Viruses in Vertebrate and Insect Cell Cultures by the Use of Hybridization Techniques.		5. REPORT DATE May 1980 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) William Meinke, D.A. Goldstein, Cynthia Alvidrez, John Spizizen, C.B. Williams, and H.R. Lukens		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Scripps Clinic and Research Foundation 10666 N. Torrey Pines Road La Jolla, California 92037		10. PROGRAM ELEMENT NO. 1EA615 11. CONTRACT/GRANT NO. Contract No. 68-02-2209
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory U.S. Environmental Protection Agency Research Triangle Park, NC 27711		13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE 600/11
15. SUPPLEMENTARY NOTES		

16. ABSTRACT

The goal of this research is to determine whether a nuclear polyhedrosis virus (NPV) can interact with a variety of mammalian and insect cells in vivo, producing either apparent or inapparent infection. The use of DNA-DNA hybridization techniques to detect the presence of viral genomes in cells will provide a sensitive method of monitoring viral persistence or replication, even in the absence of cytopathic effects or the production of infectious virus particles. It is proposed that the DNA-DNA hybridization technique be developed as a sensitive assay for the replication or persistence of NPV in vitro, allowing a more effective evaluation of the safety of these viruses as biological control agents that has been possible employing previously established methods.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Nuclear polyhedrosis virus (NPV) Viral genomes Cells in vivo		06 F,T

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS (This Report) UNCLASSIFIED 20. SECURITY CLASS (This page) UNCLASSIFIED	21. NO. OF PAGES 22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE